

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

United States Patent and Trademark
Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year)

03 September 1997 (03.09.97)

International application No.

PCT/US96/18984

Applicant's or agent's file reference

22244,0003

International filing date (day/month/year)

27 November 1996 (27.11.96)

Priority date (day/month/year)

01 December 1995 (01.12.95)

Applicant

APICELLA, Michael, A. et al

1. The designated Office is hereby notified of its election made:



in the demand filed with the International Preliminary Examining Authority on:

23 June 1997 (23.06.97)



in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was



was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer

Beatriz Morariu

Facsimile No.: (41-22) 740.14.35

Telephone No.: (41-22) 338.83.38

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PATENT COOPERATION TREATY

PCT

COMMUNICATION OF
INTERNATIONAL APPLICATIONS

(PCT Article 20)

From the INTERNATIONAL BUREAU

To:

United States Patent and Trademark
Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

Date of mailing:

18 September 1997 (18.09.97)

in its capacity as designated Office

The International Bureau transmits herewith copies of the international applications having the following international application numbers and international publication numbers:

International application no.:

PCT/US96/18984 ✓

International publication no.:

WO97/19688

**CORRECTED VERSION
VERSION CORRIGEE**

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra
Telephone No.: (41-22) 338.83.38

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The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ _____

PCT

DEMAND

CHAPTER II

under Article 31 of the Patent Cooperation Treaty:

The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty.

For International Preliminary Examining Authority use only

Identification of IPEA		Date of receipt of DEMAND	
Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION		Applicant's or agent's file reference 22244.0003	
International application No. PCT/US96/18984	International filing date (day/month/year) 27 November 1996	(Earliest) Priority date (day/month/year) 1 December 1995	
Title of invention Non-Toxic Mutants of Pathogenic Gram-Negative Bacteria			
Box No. II APPLICANT(S)			
Name and address: (Family Name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) University of Iowa Research Foundation 100 Oakdale Campus, #214 TIC Iowa City, Iowa 52242-5000 United States of America		Telephone No.: (319) 335-4548	
		Facsimile No.: (319) 335-4489	
		Teleprinter No.:	
State (i.e. country) of nationality: United States of America		State (i.e. country) of residence: United States of America	
Name and address: (Family Name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) The Regents of the University of California 300 Lakeside Drive, 22nd Floor Oakland, California 94612-3550 United States of America			
State (i.e. country) of nationality: United States of America		State (i.e. country) of residence: United States of America	
Name and address: (Family Name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) American Cyanamid Company Five Giralda Farms Madison, New Jersey 07940 United States of America			
State (i.e. country) of nationality: United States of America		State (i.e. country) of residence: United States of America	
<input type="checkbox"/> Further applicants are indicated on a continuation sheet.			

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Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative

and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.
☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.
☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family Name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Telephone No.:
(716) 856-4000

Nelson, M. Bud
Bean, Edwin, T., Jr.
Linihan, Martin G.
Scalise, Michael F.
Johnson, Christine

Hodgson, Russ, Andrews, Woods & Goodyear
1800 One M&T Plaza
Buffalo, New York 14203
United States of America

Facsimile No.:
(716) 849-0349

Teleprinter No.:

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV STATEMENT CONCERNING AMENDMENTS

The applicant wishes the International Preliminary Examining Authority*

- (i) ☐ to start the international preliminary examination on the basis of the international application as originally filed.
- (ii) ☐ to take into account the amendments under Article 34 of
- ☐ the description (amendments attached).
 - ☐ the claims (amendments attached).
 - ☐ the drawings (amendments attached).
- (iii) ☐ to take into account any amendments of the claims under Article 19 filed with the International Bureau (a copy is attached).
- (iv) ☐ to disregard any amendments of the claims under Article 19 and to consider them as reversed.
- (v) ☐ to postpone the start of the international preliminary examination until the expiration of 20 months from the priority date unless that Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Box No. V ELECTION OF STATES

☒ The applicant hereby elects all eligible states *(that is, all States which have been designated and which are bound by Chapter II of the PCT)* except

(If the applicant does not wish to elect certain eligible States, the name(s) or country code(s) of those States must be indicated above.)

This Page Blank (uspto)

Box No. VI CHECKLIST

The demand is accompanied by the following documents for the purposes of international preliminary examination:

- | | | | |
|----|---|---|------------|
| 1. | amendments under Article 34 | | |
| | description | : | ___ sheets |
| | claims | : | ___ sheets |
| | drawings | : | ___ sheets |
| 2. | letter accompanying amendments under Article 34 | : | ___ sheets |
| 3. | copy of amendments under Article 19 | : | ___ sheets |
| 4. | copy of statement under Article 19 | : | ___ sheets |
| 5. | other (specify): | : | ___ sheets |

For International Preliminary
Examining Authority use only

- | received | not received |
|--------------------------|--------------------------|
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |
| <input type="checkbox"/> | <input type="checkbox"/> |

The demand is also accompanied by the item(s) marked below:

- | | |
|--|--|
| 1. <input type="checkbox"/> separate signed power or attorney | 4. <input checked="" type="checkbox"/> fee calculation sheet |
| 2. <input type="checkbox"/> copy of general power of attorney | 5. <input type="checkbox"/> other (specify): _____ |
| 3. <input type="checkbox"/> statement explaining lack of signature | |

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).


M. Bud Nelson, Agent

For International Preliminary Examining Authority use only

- | | |
|----|--|
| 1. | Date of actual receipt of DEMAND: |
| 2. | Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b): |
| 3. | <input type="checkbox"/> The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. <input type="checkbox"/> The applicant has been informed accordingly. |
| 4. | <input type="checkbox"/> The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5. |
| 5. | <input type="checkbox"/> Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82. |

For International Bureau use only

Demand received from IPEA on:

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PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:

M. BUD NELSON
HODGSON, RUSS, ANDREWS,
WOODS & GOODYEAR
1800 ONE M&T PLAZA
BUFFALO NY 14203-2391

NOTIFICATION OF RECEIPT OF DEMAND

(PCT Rule 61.1(b), first sentence
and Administrative Instructions, Section 601)

Date of mailing
(day/month/year)

14 AUG 1997

Applicant's or agent's file reference
22244.0003

IMPORTANT NOTIFICATION

International application No.

PCT/US96/18984

International filing date (day/month/year)

27 NOV 96

Priority date (day/month/year)

01 DEC 95

Applicant

UNIVERSITY OF IOWA RESEARCH FOUNDATION

1. The applicant is hereby notified that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

IPEA/US 23 JUN 1997

2. This date of receipt is:

- ☒ the actual date of receipt of the demand.
☐ the date on which the proper corrections to the demand were timely received.

3. ☐ This date is **AFTER** the expiration of 19 months from the priority date.

Attention: The election(s) made in the demand does (do) not have the effect of postponing the commencement of the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22).

For details, see Annex B to Form PCT/IB/301 sent by the International Bureau and Volume II of the PCT Applicant's Guide.

- ☐ This notification confirms the information given in person or by telephone on:

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/US
Assistant Commissioner for Patents
Box PCT
Washington, D.C. 20231
Facsimile No.

Attn: IPEA/US

Authorized officer

A. Smith
Angela Smith
PCT International Division
Telephone No. (703) 305-3166

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PATENT COOPERATION TREATY

PCT

REC'D 21 APR 1998

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 22244.0003	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US96/18984	International filing date (day/month/year) 27 NOVEMBER 1996	Priority date (day/month/year) 01 DECEMBER 1995
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant UNIVERSITY OF IOWA RESEARCH FOUNDATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 6 sheets.
- ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).
- These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 23 JUNE 1997	Date of completion of this report 23 MARCH 1998
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer V. RYAN Telephone No. (703) 308-0196

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US96/18984

L Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments):*

☐ the international application as originally filed.

☒ the description, pages (See Attached), as originally filed.

pages _____, filed with the demand.

pages _____, filed with the letter of _____.

pages _____, filed with the letter of _____.

☒ the claims, Nos. (See Attached), as originally filed.

Nos. (See Attached), as amended under Article 19.

Nos. _____, filed with the demand.

Nos. _____, filed with the letter of _____.

Nos. _____, filed with the letter of _____.

☒ the drawings, sheets/fig (See Attached), as originally filed.

sheets/fig _____, filed with the demand.

sheets/fig _____, filed with the letter of _____.

sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☒ the description, pages NONE.

☒ the claims, Nos. NONE.

☒ the drawings, sheets/fig NONE.

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

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Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 11

Claims 16-18, 22, 23, 25, 27, and 28 lack novelty under PCT Article 33(2) as being anticipated by Lee et al. Lee et al (Journal of Biological Chemistry 270(45):27151-27159, 1995) disclose a htrB mutant of *Haemophilus influenzae* Nontypable strain 2019 and a method of making the mutant endotoxin.

Thus, the prior art disclosure is viewed as anticipating the claimed invention.

Claims 23-25 lack novelty under PCT Article 33(2) as being anticipated by any one of Munford et al or Myers et al.

Munford et al (US Patent #4,929,604) teach a composition comprised of a mutant lipopolysaccharide with reduced toxicity that was administered to rabbits. (See especially columns 9 and 10)

Myers et al (US Patent #4,912,094) teach a composition comprised of modified lipopolysaccharide which is less toxic and maintains its antigenic and immuno-stimulating properties. (See especially Abstract)

While the lipopolysaccharide of the prior art references were not obtained from a htrB mutant, they nevertheless appear to be the same or an obvious or analogous variant of the proteins broadly and non-specifically claimed by applicants because they appear to possess the same or similar functional characteristics. The burden is upon applicants to show an unobvious distinction between the material structural and functional characteristics of the claimed endotoxin and the endotoxin of the prior art. See *In re Best*, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and *In re Fitzgerald et al.*, 205 USPQ 594.

Thus, the prior art disclosure is viewed as anticipating the claimed invention.

Claims 1, 2, 4, 5, 7, 8, 10, 11, 15-19, and 21-29 lack an inventive step under PCT Article 33(3) as being obvious over Lee et al in view of Myers et al.

Lee et al (Journal of Biological Chemistry 270(45):27151-27159, 1995) teach a method of producing mutant endotoxin from a htrB mutant strain of *Haemophilus influenzae*. Lee et al also teach that the mutation results in an O-deacylated endotoxin. Lee et al, however, differ in not teaching the administration of the endotoxin.

Myers (US Patent #4,912,094) teach that de-O-acylated endotoxin can be used as a vaccine against gram-negative infections. The endotoxin can be administered in saline, in a lipid emulsion system, or in an oil-in-water emulsion.

Given the teachings of the prior art that a mutation in the htrB gene can result in the production of a O-deacylated endotoxin, and de-O-acylated endotoxins can be administered as vaccines against gram-negative infections, it would have been obvious to one of ordinary skill in the art at the time the invention was made to similarly administer the O-deacylated endotoxin produced from the htrB mutant with a reasonable expectation of producing a non-toxic pharmaceutical composition.

Claims 3, 9, 12, 14, and 20 meet the criteria set out in PCT Article 33(2)-(4) because the prior art does not teach or fairly suggest a method of immunizing an individual with a vaccine formulation comprised of an htrB mutant of a gram-negative bacterial pathogen.

NEW CITATIONS

LEE et al. Mutation of the htrB Locus of *Haemophilus influenzae* Nontypable Strain 2019 Is Associated with Modification of Lipid A and Phosphorylation of the Lipo-oligosaccharide. *Journal of Biological Chemistry*. 10 November 1995. Vol. 270, No. 45, pages 27151-27159. See especially Abstract.

KAROW et al. Isolation and Characterization of the *Escherichia coli* htrB Gene Whose Product is Essential for Bacterial Viability above 33 degrees C in Rich Media. *Journal of Bacteriology*. January 1991. Vol. 173, No. 2, pages 741-750. See especially page 742.

KAROW et al. The Lethal Phenotype Caused by Null Mutations in the *Escherichia coli* htrB Gene is Suppressed by Mutations in the aceBC Operon, Encoding Two Subunits of Acetyl Coenzyme A Carboxylase. *Journal of Bacteriology*. November 1992. Vol. 174, No. 22, pages 7407-7418. See especially pages 7409-7414.

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US96/18984

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. STATEMENT**

Novelty (N)	Claims	(Please See supplemental sheet)	YES
	Claims	(Please See supplemental sheet)	NO
Inventive Step (IS)	Claims	(Please See supplemental sheet)	YES
	Claims	(Please See supplemental sheet)	NO
Industrial Applicability (IA)	Claims	(Please See supplemental sheet)	YES
	Claims	(Please See supplemental sheet)	NO

2. CITATIONS AND EXPLANATIONS

The Examiner acknowledges the Applicant's response to the IPEA/408 submitted January 21, 1998.

The Applicant asserts that, by the definition of Gram-negative bacterial pathogen provided in the specification, the claims do not encompass *Escherichia coli*.

The Examiner has considered the Applicant's response concerning this issue. However, the Examiner is not persuaded because the claims are directed to Gram-negative bacterial pathogens. And, although Applicant's definition provided in the specification includes several bacterial, but does not specifically recite *Escherichia coli*, it is well known in the art that *Escherichia coli* is a gram-negative bacteria which cause diseases such as traveler's diarrhea, sepsis, and meningitis.

Applicant contends that Lee et al does not disclose htrB mutants having reduced toxicity or the use of the mutants for generating antibodies or in vaccines.

Applicant's arguments are not persuasive because, as Applicant noted, Lee et al disclose the isolation of htrB mutants of *H. influenzae*. Lee et al also disclose that LOS from the mutants does not bind to mAb 6E4, and has reduced phosphoethanolamine content and reduced acyl chains on lipid A, and migrates faster than the LOS from wild type NTHi 2019 on SDS-PAGE. Thus, the mutants appear to be the same and the reduced toxicity is viewed as inherent. Furthermore, the term "use of the mutants for generating antibodies or vaccines" is viewed as an intended use for the composition. The burden is on Applicant to show a novel or unobvious difference between the claimed product and the product of the prior art.

Applicant also contends that none of the cited references disclose the endotoxin of the present invention, nor the means by which the endotoxin or htrB mutants are made.

Applicant's arguments are not persuasive because the claims are directed to a mutant endotoxin of substantially reduced toxicity which has a phenotype of a htrB mutant (i.e., the mutant lacks one or more secondary acyl chains of lipid A contained in the (Continued on Supplemental Sheet.)

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VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Applicant asserts that Example 5 of the specification is directed to the use of htrB mutants as immunogens, and endotoxin isolated from the htrB mutants was used to immunize mice. Applicant also submits that the data presented was indicative of antibodies possessing bactericidal activity against the wild strain NTHi 2019.

The claims are directed to a vaccine and a method for immunizing an individual to prevent diseases caused by a gram-negative bacterial pathogen. However, the description does not enable any person skilled in the art to use the invention commensurate in scope with the claims. The art of vaccinating and preventing gram-negative infections is unpredictable, and there are no vaccines for many of the diseases caused by pathogens such as *Neisseria gonorrhoea*, *Neisseria meningitidis*, *Yersinia pestis*, *Brucella*, and *Moraxella catarrhalis*. Although Example 5, and the other examples provided in the specification teach that *H. influenzae* htrB mutants can be used as immunogens, the specification does not indicate that the composition provides protection against the pathogen. For example, page 26 of the specification indicates that the mutant LOS was used to immunize animals, and mean antibody titers are provided. However, there is no correlation between the in vitro antibody titer results and prevention of disease, and the specification does not teach that any particular antibody level is indicative of protection.

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US96/18984

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(6): A61K 35/66, 39/00, 39/02; C07K 14/195 and US Cl.: 424/9.2, 184.1, 234.1, 236.1; 530/403, 825

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
pages, 1-63, as originally filed.
pages, NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the claims,
numbers, 1-26, as originally filed.
numbers, NONE, as amended under Article 19.
numbers, NONE, filed with the demand.
and additional amendments:
Claims 27-29, filed with the letter of 21 January 1998.

This report has been drawn on the basis of the drawings,
sheets, 1-8, as originally filed.
sheets, NONE, filed with the demand.
and additional amendments:
NONE

V. 1. REASONED STATEMENTS:

The report as to Novelty was positive (YES) with respect to claims 1-15, 19, 20, 21, 24, 26, and 29.
The report as to Novelty was negative (NO) with respect to claims 16-18, 22, 23, 25, 27, and 28.
The report as to Inventive Step was positive (YES) with respect to claims 3, 6, 9, 12, 14, and 20.
The report as to Inventive Step was negative (NO) with respect to claims 1-2, 4-5, 7-8, 10-11, 15-19, 21-29.
The report as to Industrial Applicability was positive (YES) with respect to claims 1-29.
The report as to Industrial Applicability was negative (NO) with respect to claims NONE.

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):

wild type gram-negative bacterial pathogen). Munford et al disclose an endotoxin with reduced toxicity and without non-hydroxylated acyl chains attached to the lipid A. Myers et al disclose modified lipopolysaccharides, particularly de-3-O-acylated monophosphoryl lipid A and de-3-O-acylated diphosphoryl lipid A, which are less toxic. The rejection pertaining to Zollinger et al is withdrawn.

Applicant also contends that the references do not teach the use of htrB mutants to generate antibodies or in vaccines. Applicant's arguments are not persuasive because the Lee et al reference is cited to show a method of producing O-deacylated mutant endotoxins from a htrB mutant strain of *H. influenzae*. Myers et al is combined with the primary reference to show that O-deacylated endotoxins can be used as a vaccine against gram-negative infections. Therefore, the combined teachings of the prior art suggests that O-deacylated endotoxins produced from the htrB mutant strain can also be administered as a vaccine preparation.

Claim 16, and 17 lack novelty under PCT Article 33(2) as being anticipated by Karow et al (1991). Karow et al (Journal of Bacteriology 173(2):741-750, 1991) disclose a htrB mutant of *Escherichia coli*. The term "vaccine" is viewed as an intended use for the bacteria. Thus, the prior art disclosure is viewed as anticipating the claimed invention. Claims 16-18 lack novelty under PCT Article 33(2) as being anticipated by Karow et al (1992). Karow et al (Journal of Bacteriology 174(22):7407-7418, 1992) disclose a htrB mutant of *Escherichia coli*. The term "vaccine" is viewed as an intended use of the bacteria. Thus, the prior art disclosure is viewed as anticipating the claimed invention.

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causes a phenotype of a resultant *htrB* mutant characterized by endotoxin lacking at least one secondary acyl chain on lipid A contained in the wild type gram-negative bacterial pathogen.

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28. A mutant gram-negative bacterial pathogen of substantially reduced toxicity, made according to the method of claim 27, wherein the gram-negative bacterial pathogen having substantially reduced toxicity is used to generate endotoxin-specific antibodies.

10

29. A method for producing endotoxin-specific antisera for a use selected from the group consisting of in diagnostic assays, and for passive immunization, the method comprises immunizing an individual with a vaccine formulation comprising an active ingredient selected from the group consisting of an *htrB* mutant of said gram-negative bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, and endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen said endotoxin conjugated to a carrier protein; and collecting antibody produced from said immunized individual; wherein said *htrB* mutant lacks one or more secondary acyl chains of lipid A contained in the wild type gram-negative bacterial pathogen resulting in substantially reduced toxicity when compared to lipid A of the wild type gram-negative bacterial pathogen.

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PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: M. BUD NELSON
HODGSON, RUSS, ANDREWS, WOODS &
GOODYEAR
1800 ONE M&T PLAZA
BUFFALO, NEW YORK 14203

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing
(day/month/year) 15 APR 1998

Applicant's or agent's file reference
22244.0003

IMPORTANT NOTIFICATION

International application No.
PCT/US96/18984

International filing date (day/month/year)
27 NOVEMBER 1996

Priority Date (day/month/year)
01 DECEMBER 1995

Applicant
UNIVERSITY OF IOWA RESEARCH FOUNDATION

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.
4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer
V. RYAN

Telephone No. (703) 308-0196

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PCT

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The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:

☒ agent ☐ common representative

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The priority of the following earlier application(s) is hereby claimed:

Country (in which, or for which, the application was filed)	Filing Date (day/month/year)	Application No.	Office of filing (only for regional or international application)
item (1) United States of America	01 December 1995	08/565,943	
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 2. description : 63 sheets
 (59=description; 4=sequence listing)
 3. claims : 6 sheets
 4. abstract : 1 sheets
 5. drawings : 8 sheets

Total : 83 sheets

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Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.

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Hodgson, Russ, Andrews, Woods & Goodyear

By: M. Bud Nelson

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(21) International Application Number: PCT/US96/18988 (22) International Filing Date: 27 November 1996 (27.11.96) (30) Priority Data: 08/565,943 01 Jan 98 / 30 mos 1 December 1995 (01.12.95) US (71) Applicants (for all designated States except US): UNIVERSITY OF IOWA RESEARCH FOUNDATION [US/US]; 100 Oakdale Campus #214 TIC, Iowa City, IA 52242-5000 (US). THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US). AMERICAN CYANAMID COMPANY [US/US]; Five Giralda Farms, Madison, NJ 07940 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): APICELLA, Michael, A. [-/US]; 2646 Johnsons Crossing, N.E., Solon, IA 52333 (US). SUNSHINE, Melvin, G. [-/US]; 340 Raven Street, Iowa City, IA 52245 (US). LEE, Na-Gyong [-/-]; Apartment 27-1309, Nam-gu Hakik-2-dong, Sindong-a, Incheon (KR). VARUMUGHAM, Rasappa [-/US]; 15 Elatia Circle, Pittsford, NY 14534 (US). GIBSON, Bradford, W. [-/US]; 1324 Peralta Avenue, Berkeley, CA 94702 (US).		(74) Agents: NELSON, M., Bud et al.; Hodgson, Russ, Andrews, Woods & Goodyear, 1800 One M & T Plaza, Buffalo, NY 14203-2391 (US). (81) Designated States: AU, CA, JP, KR, MX, NZ, US, Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: NON-TOXIC MUTANTS OF PATHOGENIC GRAM-NEGATIVE BACTERIA (57) Abstract <p>A method is provided for identifying, isolating, and producing <i>htrB</i> mutants of gram-negative bacterial pathogens. The method comprises mutating the <i>htrB</i> gene of a gram-negative bacterial pathogen so that there is a lack of a functional <i>htrB</i> protein, resulting in a mutant that lacks one or more secondary acyl chains contained in the wild type gram-negative bacterial pathogen, and displays substantially reduced toxicity as compared to the wild type strain. Also, the present invention provides methods for using a vaccine formulation containing the <i>htrB</i> mutant, the endotoxin isolated therefrom, or the endotoxin isolated therefrom which is then conjugated to a carrier protein, to immunize an individual against infections caused by gram-negative bacterial pathogens by administering a prophylactically effective amount of the vaccine formulation.</p>		

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NON-TOXIC MUTANTS OF PATHOGENIC GRAM-NEGATIVE BACTERIA

This invention has been made with government support under grant AI 24616 awarded by the National Institutes of Health. The government has certain rights
5 in the invention.

Field of the Invention

The present invention relates to compositions comprising altered endotoxin (lipooligosaccharide (LOS);
10 lipopolysaccharide (LPS)) of gram-negative bacterial pathogens. More particularly, the present invention relates to the making of a form of endotoxin, by a genetically engineered gram-negative pathogen, which lacks a substantially toxic lipid A portion. Also
15 disclosed are prophylactic and therapeutic uses of the substantially detoxified endotoxin, and of mutant gram-negative bacteria producing the substantially detoxified endotoxin.

20 Background of the Invention

Gram-negative bacteria have an outer membrane comprised of components including proteins,
lipoproteins, phospholipids, and glycolipids. The glycolipids comprise primarily endotoxin-lipopoly-
25 saccharides (LPS) or lipooligosaccharides (LOS), depending on the genus of bacteria. LPS are molecules comprised of
a) a lipid A portion which consists of a glucosamine disaccharide that is substituted with phosphate groups
30 and long chain fatty acids in ester and amide linkages;
b) a core polysaccharide which is attached to lipid A by an eight carbon sugar, KDO (ketodeoxyoctonate), and heptose, glucose, galactose, and N-acetylglucosamine;
and

c) an O-specific side chain comprised of repeating oligo-saccharide units which, depending on the genera and species of bacteria, may contain mannose, galactose, D-glucose, N-acetylgalactosamine, N-acetylglucosamine, L-rhamnose, and a dideoxyhexose (abequose, colitose, tyvelose, paratose, trehalose). LOS has a similar structure as LPS, containing a lipid A portion and a complex carbohydrate structure, but differs in that it does not contain repeating O-side chains.

10 The major antigenic determinants of gram-negative bacteria are believed to reside in the carbohydrate structure of the O-specific side chain of LPS and the complex carbohydrate structure of LOS. These carbohydrate structures may vary for different species of the same genera of gram-negative bacteria by varying one or more of the sugar composition; the sequence of oligosaccharides; the linkage between the oligosaccharides; and substitutions/modifications of an oligosaccharide (particularly a terminal oligosaccharide).

20 LPS and LOS have been considered as bacterial components which have potential as vaccine immunogens because of the antigenic determinants ("epitopes") residing in their carbohydrate structures. However, the chemical nature of LPS and LOS prevent the use of these molecules in vaccine formulations; i.e., active immunization with LPS or LOS is unacceptable due to the inherent toxicity of the lipid A portion. The patho-physiologic effects induced (directly or indirectly) by lipid A of LPS or LOS in the bloodstream include fever; leucopenia; leucocytosis; the Schwartzman reaction; disseminated intravascular coagulation; abortion; and in larger doses, shock and death. Accordingly, there are no currently available vaccines which induce antibody responses to LPS or LOS epitopes.

As shown in FIG. 1, the lipid A portion of endotoxin generally comprises a hydrophilic backbone of glucosamine disaccharide which is either monophosphorylated or diphosphorylated (positions 1 and 4'); and which carries at least six molecules of ester- and amide-bound fatty acids. Four molecules of (R)-3-hydroxytetradecanoate (e.g. 3-hydroxy-myristoyl or β -hydroxymyristic acid or β -OH) are linked directly to the lipid A backbone at positions 2, 3, 2', and 3'. Hydroxyl groups of two of the four molecules of β -OH are substituted with normal fatty acids (termed "secondary acyl chains", and including dodecanoate, tetradecanoate, and hexadecanoate) in forming acyloxyacyl groups.

One approach to making a detoxified endotoxin molecule involves isolating the endotoxin, and enzymatically-treating the isolated endotoxin with a human neutrophilic acyloxyacyl hydrolase (U.S. Patent Nos. 4,929,604, 5,013,661 and 5,200,184). The acyloxyacyl hydrolase hydrolyzes the fatty acids (non-hydroxylated, secondary acyl chains) from their ester linkages to hydroxy groups of β -OH (hydroxylated). The resultant altered endotoxin, from enzymatic treatment, contained a lipid A moiety lacking non-hydroxylated fatty acids. This altered endotoxin exhibited reduced *in vivo* toxicity, but retained antigenicity.

Another approach involves a method of modifying isolated endotoxin by selectively removing the β -OH that is ester-linked to the reducing-end glucosamine backbone at position 3 (U.S. Patent No. 4,912,094; Reexamination B1:4,912,094). The selective removal of β -OH was accomplished using alkaline hydrolysis. The resultant modified endotoxin exhibited reduced *in vivo* toxicity, but retained antigenicity.

Both approaches involve chemically treating isolated endotoxin. Neither approach discloses the production in a gram negative bacterial pathogen of an

endotoxin having substantially reduced toxicity, yet retaining antigenicity. Further, there has been no disclosure of the use of a gram-negative bacteria, which has been engineered to produce an endotoxin having

5 substantially reduced toxicity and yet retaining antigenicity, in a prophylactic or therapeutic vaccine against endotoxic shock and gram-negative bacteremia.

Summary of the Invention

10 The present invention is directed to a method for producing, in a mutant gram-negative pathogen, LPS or LOS which exhibits substantially reduced toxicity as compared to the wild type endotoxin, and which retains the antigenicity of its corresponding wild type

15 endotoxin. The method comprises creating a mutation in the *htrB* gene of the gram-negative bacterial pathogen such that there is a lack of functional HtrB protein in the mutated gram-negative bacterial pathogen. It was found that lipid A produced by the *htrB* mutant lacks one

20 or both of the fatty acids (non-hydroxylated or secondary acyl chains) thereby rendering the endotoxin in an isolated form, or the mutant gram-negative bacterial pathogen bearing the endotoxin, substantially reduced in toxicity and yet retaining antigenicity, as

25 compared to wild type. Endotoxin isolated from *htrB* mutants, or the *htrB* mutants themselves (whole cell vaccine), can be used to immunize individuals at risk of gram-negative bacteremia by inducing antibodies to the major antigenic determinants which reside in the

30 carbohydrate structure of the O-specific side chain of LPS and the complex carbohydrate structure of LOS. Further, the *htrB* mutants can be engineered to express heterologous antigens of other microbial pathogens at the surface of the *htrB* mutant for presentation to a

35 vaccinated individual's immune system in a multivalent vaccine. Also, the endotoxin isolated from the *htrB*

mutants of the present invention may be used to generate LPS or LOS-specific antibody which may be useful for passive immunization and as reagents for diagnostic assays directed to detecting the presence of gram-negative bacterial pathogens in clinical specimens.

Brief Description of the Figures

- FIG. 1 is a schematic representation of the general structure of lipid A of gram negative bacteria of the family *Enterobacteriaceae*.
- FIG. 2A is a schematic representation of the general structure of a species of lipid A, from the LOS of an *htrB* mutant, comprising pentaacyl diphosphoryl lipid A.
- FIG. 2B is a schematic representation of the general structure of a species of lipid A, from the LOS of an *htrB* mutant, comprising tetraacyl diphosphoryl lipid A.
- FIG. 3 is a graph showing the relative toxicity of an *htrB* mutant (O, Δ) as compared to wild type bacteria (\square) in a TNF α release assay.
- FIG. 4 is a photograph showing human primary respiratory epithelial cells unstimulated (control), exposed to NTHi 2019 LOS, or exposed to *htrB* mutant B29 LOS, and reacted with either a fluorescent probe that hybridizes to TNF α mRNA (probe 1) or a fluorescent control probe (probe 2).
- FIG. 5 is a graph showing mean titers of anti-LOS antibody against NTHi 2019 LOS (antigen coating) in ELISA from mice immunized with NTHi 2019 (Pool 595), *htrB* mutant B29 LOS (Pool 597), or *htrB* mutant B29 LOS conjugated to a carrier protein (Pool 606), with adjuvant.
- FIG. 6 is a graph showing the mean titers of anti-LOS antibody against *htrB* mutant B29 LOS (antigen coating) in ELISA from mice immunized with NTHi 2019 (Pool 595), *htrB* mutant B29 LOS (Pool 597), or *htrB* mutant B29 LOS conjugated to a carrier protein (Pool 606), with adjuvant.

FIG. 7 is a schematic representation comparing the structures of wild type *Salmonella* lipid A and *htrB* mutant lipid A.

5

Detailed Description of the Invention

Definitions:

10 "Endotoxin" is a term used herein for purposes of the specification and claims to refer to the LPS or LOS of gram-negative bacterial pathogens, wherein the endotoxin is either in a cell-associated or isolated form. "*htrB* endotoxin" and "*htrB* mutant endotoxin" refer to endotoxin isolated and purified from an gram-negative
15 bacterial pathogen *htrB* mutant.

"vaccine candidate or vaccine antigen" is a term used herein for purposes of the specification and claims to refer to an endotoxin epitope having one or more of the following properties (a-d): (a) is immunogenic; (b)
20 is surface-exposed (which can be shown by techniques known in the art including immunofluorescence assays, electron microscopy staining procedures, and by bactericidal assays); (c) induces antibody having bactericidal activity in the presence of complement
25 and/or functions in immune clearance mechanisms; (d) induces antibody which neutralizes other functional activity of the epitope (immunogenicity, or toxicity, etc.).

"Gram-negative bacterial pathogen" is a term used
30 herein for the purposes of the specification and claims to refer to one or more pathogenic (to humans or animals) bacterium of a genus and species including *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Haemophilus ducreyi*, other
35 *Haemophilus* species, *Moraxella catarrhalis*, *Campylobacter jejuni*, *Salmonella typhimurium*, other

Salmonella species, *Shigella dysenteriae*, and other *Shigella* species, and *Pseudomonas aeruginosa*.

"Substantially reduced in toxicity" is a term used herein for the purposes of the specification and claims to refer to a reduction in bioactivity of at least 10 fold to 100 fold or more as compared to wild type endotoxin.

"Carrier protein" is a term used herein for the purposes of the specification and claims to refer to a protein which is conjugated to the *htrB* mutant endotoxin. While the *htrB* mutant endotoxin appears to be immunogenic on its own, it is known in the art that conjugation to a carrier protein can facilitate immunogenicity. Proteins which may be utilized according to the invention include any protein which is safe for administration to mammals and which may serve as an immunologically effective carrier protein. In particular embodiments, cell surface proteins, membrane proteins, toxins and toxoids may be used. Criteria for safety would include absence of primary toxicity and minimal risk of allergic reaction. Diphtheria and tetanus toxoids fulfill these criteria; that is, suitably prepared they are non-toxic, and the incidence of allergic reactions is acceptably low. Although the risk of allergic reaction may be significant for adults, it is minimal for infants.

According to additional particular embodiments of the invention, appropriate carrier proteins include, but are not limited to *Salmonella* flagellin, *Haemophilus* pilin, *Pseudomonas pili*, *Pseudomonas* exotoxin, outer membrane proteins of *Haemophilus* (15 kDa, 28-30 kDa, and 40 kDa membrane proteins) or *N. meningitidis* or *N. gonorrhoeae*, *Escherichia coli* heat labile enterotoxin LT_B, cholera toxin, pneumolysin of *S. pneumoniae*, and viral proteins including rotavirus VP7 and respiratory syncytial virus F and G proteins. Additionally, there

are many carrier proteins known in the art including, but not limited to, keyhole limpet hemocyanin, bovine serum albumin, and diphtheria toxin cross-reactive mutant protein ("CRM"). Additionally, there are several methods known in the art for conjugating endotoxin to a carrier protein. Such methods may include, but are not limited to, the use of glutaraldehyde, or succinimidyl *m*-maleimidobenzoate, or 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide, or by using bromoacetylated carrier protein (see, e.g. Robey et al., 1989, *Anal. Biochem.* 177:373-377). Conjugation of *htrB* endotoxin to a carrier protein toxin may reduce toxicity of the carrier protein toxin, but residual toxicity may remain. Further detoxification may be accomplished by means known in the art such as employing formalin which reacts with free amino groups of the carrier protein toxin.

Alternatively, native carrier protein toxin may be detoxified with formalin to produce a conventional toxoid before conjugation to the *htrB* mutant endotoxin. However, the prior formalin treatment reduces the number of free amino groups available for reaction during the conjugation process. CRM have an advantage in that they have no inherent toxicity yet none of their amino groups are occupied by the formalin. In the case of CRM197, which is immunologically identical to native toxin, treatment with formalin (though there is no need to detoxify) greatly enhances the immunological response. It is thought that this is due to stabilization of the molecule against degradation by mechanisms of the body and/or aggregation by cross-linking (immunogenicity of particles increases with size). While tetanus and diphtheria toxins are desirable carrier proteins, there may be other candidate carrier proteins which may also be suitable. Such other candidates for carriers include toxins and proteins of pseudomonas, staphylococcus, streptococcus, pertussis, and *E. coli*.

Conjugation of endotoxin to carrier proteins can be performed by a variety of methods such as by direct conjugation to the carrier protein by cyanogen bromide, reductive amination, or by using bifunctional
5 linkers. Such bifunctional linkers include, but are not limited to, N-hydroxy succinimide-based linkers cystamine, glutaraldehyde, and diamino hexane. *htrB* endotoxin is modified to contain sulfhydryl groups with the use of N-hydroxy succinimide-based linkers, or
10 by the use of carbodiimide-mediated condensation of cystamine. The sulfhydryl-containing *htrB* endotoxin intermediates are then reacted to a carrier protein that has been derivatized with N hydroxy succinimidyl bromo acetate. Preferably, sulfhydryl groups are exposed on
15 the conjugating *htrB* endotoxin for making a thiol linkage with the bromoacetylated carrier protein.

In a specific embodiment of the invention, *htrB* mutant endotoxin may be conjugated to a carrier protein, such as CRM, by using long chain sulfo N-succinimidyl 3-
20 (2-pyridylthio)-propionate to thiolate the primary amino group(s) of the endotoxin. Long chain sulfo N-succinimidyl 3-(2-pyridylthio)-propionate was added to approximately 13 mg of the saccharide component of the
30 *htrB* endotoxin in 0.1 M NaHCO₃ pH7.0 at a ratio of 1:1 (w/w) and incubated for an hour at room temperature. The mixture is then purified by gel filtration. The long chain sulfo N-succinimidyl 3-(2-pyridylthio)-propionate derivatized fractions were pooled. The N-pyridyl disulfides present in the derivatized fractions were
35 reduced with 100 mM dithiothreitol, and purified by gel filtration. The thiolated endotoxin fractions were then collected. CRM197 was bromoacetylated by adding bromoacetic acid N hydroxy succinimide in a small volume of dimethyl formamide dropwise to CRM (in 0.1 M NaHCO₃)
40 at a ratio of 1:1 (w/w) at 4°C. The solution was mixed and incubated for 1 hour at room temperature. The

reaction mixture was then purified by gel filtration, and the fractions containing bromoacetylated protein were collected. Derivatization of amino groups on carrier protein to bromoacetyl groups was monitored by a decrease in the amount of free amino groups.

5 Bromoacetyl CRM in 0.1 M NaHCO₃ was added to the thiolated *htrB* mutant endotoxin at a 1:1.5 ratio of protein to endotoxin (w/w) in 0.1 M NaHCO₃/1 mM EDTA, and the reaction was incubated overnight at 4°C. The final

10 conjugate was then be purified by gel filtration in a phosphate buffered saline pH 6.9.

The methods and compositions of the present invention relate to LPS and LOS biosynthetic pathways of gram-negative bacterial pathogens. More specifically,

15 the present invention relates to mutations in the *htrB* gene of gram-negative bacterial pathogens resulting in mutant bacteria bearing endotoxin which is substantially reduced in toxicity, and yet retains antigenicity, as

20 compared to wild type bacteria of the same species.

The genetics of lipid A biosynthesis of enteric bacteria, as it was known at the time of the present invention, is summarized in Schnaitman and Klena (1993, *Microbiol. Rev.* 57:655-682). Genes *lpxA*, *lpxB*,

25 *lpxC*, and *lpxD* encode gene products which function on the glucosamine backbone of lipid A including transfer of β -hydroxymyristic acid to glucosamine. The *htrB* gene was described as a gene that affects the inner core structure (KDO, heptose, phosphorylethanolamine (PEA))

30 which was discovered during a screen for genes necessary for growth of *Escherichia coli* at elevated temperatures. Knockout mutations of *htrB* resulted in mutant *E. coli* which exhibited a reduced sensitivity to deoxycholate, an inability to grow at temperatures above 32.5°C, and a

35 decrease in LPS staining intensity (Schnaitman et al., 1993, *supra*; Karow et al., 1992, *J. Bacteriol.* 174:7407-

7418). Karow et al. further noted that at between about 30°C to about 42°C, *E.coli htrB* mutants have changes in the fatty acid composition of both LPS and phospholipids, and particularly, overproduce
5 phospholipids, as compared to wild type. However, it was neither known nor suggested which one or more of the at least six molecules of ester- or amide bound fatty acids is lacking in the lipid A portion of LPS of *htrB* mutants. Also no mention was made that *htrB* mutants
10 contained a lipid A moiety specifically lacking one or both non-hydroxylated (secondary acyl chain) fatty acids responsible for endotoxicity; i.e. that the *htrB* mutant contained an altered endotoxin exhibiting reduced *in vivo* toxicity, but retaining antigenicity ("*htrB*
15 endotoxin"), as compared to wild type.

The discoveries comprising the present invention include the unexpected results that knockout mutations of the *htrB* gene of gram-negative bacteria (including the family *Enterobacteriaceae*) result in *htrB*
20 mutants which specifically lack one or more secondary acyl chain fatty acids which are ester-bound to the hydroxyl groups of two of the four molecules of β -OH (as shown in FIG. 2). Thus, it appears that the HtrB protein has either acyltransferase activity, or
25 indirectly or directly affects regulation of acyltransferase activity. The following examples are presented to illustrate preferred embodiments of aspects of the present invention, and are not intended to limit the scope of the invention. In particular, a preferred
30 embodiment is the making of an *H. influenzae htrB* mutant, and methods of using the same as a whole cell, or to isolate therefrom the endotoxin, in vaccine preparations or to generate antibodies for therapeutic or diagnostic applications. However, since the lipid A
35 moiety is highly conserved among bacteria of the family *Enterobacteriaceae* and closely related gram-negative

bacteria, the invention relates to gram-negative bacterial pathogens, as defined previously herein. There is microheterogeneity in terms of the length of the secondary acyl chain (12 or 14 carbon chains) and to which of the four β -OH are substituted (1, 2, or 4) (Erwin et al., 1991, *Infect Immun* 59:1881-1887); however, the nature of the substitution is the same and thus the particular steps (genes and gene products) involved in the biosynthetic pathway appear conserved. For example, removal of secondary acyl chains from various gram-negative bacterial pathogens (*E. coli*, *H. influenzae*, *P. aeruginosa*, *S. typhimurium*, and *N. meningitidis*) using human acylxyacyl hydrolase resulted in deacylated LPS from all species tested having significantly reduced mitogenic activity (Erwin et al., 1991, *supra*) as compared to the respective wild type strain.

EXAMPLE 1

20 Identification of an *htrB* gene, and generation of *htrB* mutants.

By complementing a nontypable *H. influenzae* strain 2019 with a *S. typhimurium rfaE* mutant strain, the *rfaE* gene of *H. influenzae* strain 2019 was cloned (Lee et al., 1995, *Infect Immun* 63:818-824). Sequence analysis of the upstream region of the *H. influenzae rfaE* gene revealed an open reading frame highly homologous to the *E. coli htrB* gene. The *H. influenzae htrB* gene comprises 933 bases and encodes a protein, HtrB, of 311 amino acids (SEQ ID NO:1) and an estimated molecular size of 36 kilodaltons (kDa). Comparison of the deduced amino acid sequence of the *H. influenzae* HtrB with the *E. coli* HtrB revealed shared homology (56% identity and 73% similarity). Cloning the *htrB* gene of *H. influenzae* into a plasmid, and subsequent *in vitro*

transcription-translation analysis, revealed that HtrB has an apparent molecular size of 32-33 kDa.

There are various standard techniques known to those skilled in the art for mutating a bacterial gene. Those techniques include site-directed mutagenesis, and shuttle mutagenesis using transposons. In one aspect of this embodiment, mutagenesis of the *htrB* gene was carried out by shuttle mutagenesis. A derivative of the bacterial transposon Tn3, mini-Tn3 (Seifert et al., 1986, *Proc. Natl. Acad. Sci. USA* 83:735-739), was used as an insertion sequence to mutate the *htrB* gene. A 2.4 kilobase (kb) *Bgl*III containing the *htrB* gene from *H. influenzae* was cloned into a plasmid which was used as a target for mini-Tn3 transposon mutagenesis. Briefly, introduced into a single bacterial cell (*E. coli*), is the plasmid containing the *htrB* gene; a plasmid immune to Tn3 transposition and containing transposase (which mediates the cointegration between Tn3 and the target molecules); and a plasmid containing mini-Tn3.

After allowing for transposition, the bacterial cells are mated with an *E. coli* strain containing the *cre* enzyme that is used to resolve cointegrates in shuttle mutagenesis. Transconjugates were selected for with antibiotics (kanamycin, ampicillin, and streptomycin) and analyzed by restriction endonuclease digestion.

Two plasmids, termed pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location. Each plasmid was used to transform nontypable *H. influenzae* strain 2019 and bacterial cell transformants were selected for by growth in the presence of chloramphenicol (1.5 μ g/ml), resulting in identification of mutant strains designated NTHi B28 and B29, respectively. Locations of the mTn3 insertion in the chromosomes of the NTHi mutants were

confirmed by genomic Southern hybridization using the 2.4 kb *Bgl*III fragment as a probe. In particular, a *Bgl*III digest of NTHi strain 2019 DNA resulted in a 2.4 kb fragment; whereas similar digests of DNA from mutants NTHi B28 and B29 revealed 4.0 kb fragments. Further, the 4.0 kb fragments were digested by *Eco*RI which is present in the mTn3.

Alternatively, methods are known in the art to perform site-directed mutagenesis into a bacterial gene (See for example, Halladay, 1993, *J. Bacteriol.* 175:684-692), and recombination of the mutated bacterial gene into the bacterial chromosome. A selectable kanamycin resistance cassette may be used to insert into, and mutate, the *htrB* gene contained within a shuttle plasmid. Subsequent transformation into a bacterial host cell with the shuttle plasmid, and recombination of the bacterial genome (at the site of the genomic copy of the *htrB* gene) with the cassette via *htrB* flanking sequences, results in the site-directed mutagenesis of the bacterial *htrB* gene.

Primer extension analysis can be used to determine the promoter region of the *htrB* gene. The *H. influenzae htrB*'s promoter region was determined by primer extension analysis by first growing the bacteria, harvesting and purifying the RNA, and using a commercial primer extension kit according to the manufacturer's suggestions. A single transcription site was found using a primer (SEQ ID NO:2) complementary to the 5' region of the *htrB* open reading frame. The first nucleotide was a cytosine (C) residue located at 21 bp upstream of the putative translation start site, ATG. The region upstream of the transcription start site contained a sequence (SEQ ID NO:1, bases 13 to 29) similar to the consensus -10 region of the bacterial σ^{70} -independent promoters at an appropriate distance. An

element (SEQ ID NO:1, bases 1 to 6) resembles the consensus sequence of the -35 region.

EXAMPLE 2

5 Characterization of *htrB* mutants Growth Characteristics

NTHi B28 and B29 strains were initially selected at 30°C, and were unable to grow at 37°C. With further passages at 30°C, the NTHi *htrB* mutants began to
10 lose temperature sensitivity and demonstrated a slow rate of growth, as compared to NTHi 2019, at 37°C. However, for growth temperatures greater than or equal to 38.5°C, the temperature sensitivity remained for the *htrB* mutants.

15 It was reported previously that *E. coli htrB* mutants demonstrated a change in membrane permeability to various compounds including kanamycin and deoxycholate (Karow et al., 1992, *supra*). The NTHi *htrB* mutants were also tested for sensitivity to kanamycin
20 and deoxycholate. Overnight cultures grown at 30°C were then diluted and allowed to grow in the presence of 5 µg/ml kanamycin at either 30°C or 37°C. At 30°C, no difference was detected in the growth rate between NTHi 2019 and the NTHi *htrB* mutant strains in the absence of
25 kanamycin. However, the growth of the *htrB* mutants was significantly inhibited in the presence of kanamycin, whereas NTHi 2019 was not affected. For the *htrB* mutants, the sensitivity to kanamycin was even greater at 37°C, since the mutants failed to show growth in the
30 presence of kanamycin at 37°C. Likewise, at 30°C the *htrB* mutants showed sensitivity, as compared to NTHi strain 2019, at concentrations of greater than 500 µg/ml deoxycholate, and failed to grow at 1000 µg/ml. At
35 37°C, the *htrB* mutants showed almost complete inhibition of growth in the presence of only 250 µg/ml deoxycholate.

Endotoxin Characteristics

The LPS of *E. coli htrB* mutants has been characterized as being weakly stained on silver-stained polyacrylamide gels, but its migration pattern did not vary as compared to LPS from wild type. In contrast, the LOS from NTHi mutants B28 and B29 migrated faster than that from NTHi strain 2019 on silver-stained gels. Additionally, the LOS from the B28 and B29 mutants displayed a brownish color rather than black, as displayed by NTHi 2019. Reconstitution, by introducing a plasmid with an intact *htrB* gene into the mutant, of NTHi mutant B29 confirmed that the differences in growth characteristics and LOS migration and staining were due to mutation of the *htrB* gene.

The NTHi *htrB* mutant LOS and wild type LOS were each analyzed by electrospray ionization-mass spectrometry (ESI-MS) to provide molecular mass profiles for the different components of LOS. First, LOS was isolated from the respective strains. LPS or LOS can be isolated by the phenol-water method (Westphal et al., 1965, *Methods in Carbohydrate Chemistry* 5:83-91); or using an alternative purification procedure (using a NTHi neprotease; Hitchcock et al., 1983, *J. Bacteriol.* 154:269-277). The isolated LOS species were then O-deacylated by mild hydrazine treatment (37°C for 20 minutes; see Phillips et al., 1990, *Biomed. Environ. Mass Spectrom.* 19:731-745). Analysis by ESI-MS of the different LOS species showed that while the O-deacylated LOS from NTHi mutant B29 and NTHi 2019 were similar in molecular mass profile, two differences can be clearly discerned. In the *htrB* mutant, there is a decrease (50% reduction) in the amount of LOS containing two phosphoethanolamines (PEA) in the inner core structure; and there is a shift to high molecular weight LOS species containing more hexoses. These findings suggest that the degree of phosphorylation may be affecting chain progression from

specific heptose moieties, and that HtrB either directly or indirectly affects phosphorylation of LOS.

Mass spectrometry was used to analyze the lipid A. More specifically, lipid A from *htrB* mutant LOS and from wild type LOS were each analyzed by liquid secondary ion mass spectrometry (LSIMS) in the negative ion mode to provide a spectrum of molecular ions for the different components lipid A. First, the LOS species were each hydrolyzed in 1% acetic acid for 2 hours at 100°C at a concentration of 2 mg/ml. The hydrolysates were centrifuged, and the supernatants removed. The water soluble crude lipid A fractions were washed twice in water, and once in an organic mixture (chloroform/methanol/water; by volume 2:1:1) and then evaporated to dryness. For analysis, the lipid samples were redissolved in CH₂Cl₂/CH₃OH (3:1, v/v) and 1 µl of nitro-benzylalcohol/triethanolamine (1:1, v/v) and applied as a liquid matrix onto a mass spectrometer. LSIMS of the wild type (NTHi 2019) revealed a spectrum containing two deprotonated molecular ions consistent with a hexaacyl lipid A structure containing either one (hexaacyl monophosphoryl lipid A, M_r = 1744) or two phosphates (hexaacyl diphosphoryl lipid A, M_r = 1824). This spectrum is essentially identical to that reported for the lipid A structure of LOS of *H. ducreyi* (Melaugh et al., 1992, *J. Biol. Chem.* 267:13434-13439). The lower mass fragments are believed to be ions which arise through LSIMS-induced fragmentation of higher mass mono- and diphosphorylated molecular ion species.

In contrast, the LSIMS spectrum for the lipid A preparation from the *htrB* mutant LOS lacks molecular ions corresponding to the wild type hexaacyl lipid A species. There are two high mass ions which correspond to the molecular ions for a mono- and diphosphoryl pentaacyl lipid A species missing one of the secondary acyl chains (e.g. myristic acid moiety). Further, at

the lower masses are two additional molecular ion species that correspond to a mono- and diphosphoryl tetraacyl lipid A species lacking both secondary acyl chains. In summary, the lipid A structure of the wild type's LOS is hexaacyl; whereas the lipid A structure of the *htrB* mutant shows two species, a tetraacyl (Fig. 2A) and a pentaacyl species (FIG. 2B) indicating the loss of at least one, and sometimes both secondary acyl chains. The *htrB* mutant is comprised of approximately 90% tetraacyl lipid A with only the four hydroxymyristic acid ester and amide-linked fatty acids, and approximately 10% pentaacyl lipid A with one myristic acid substitution.

15

EXAMPLE 3

Substantially reduced toxicity of *htrB* mutants

The effect due to the lack of one or more secondary acyl chains on the toxicity of a gram-negative bacterial pathogen was examined using a standard *in vitro* assay for measuring *in vivo* toxicity. Murine macrophage-like cell line J774, when stimulated by endotoxin, secretes TNF α . The amount of TNF α , a directly proportional to the toxicity of the stimulating LPS or LOS, can be measured by (a) removing the cell-free supernatant containing the TNF α ; (b) adding the supernatant to a TNF α -sensitive cell line, such as WEHI 164; and (c) measuring the resultant cytotoxicity (See for example, Espevik et al., 1986, *J Immunol Methods* 95:99; Sakurai et al., 1985, *Cancer Immunol Immunother* 20:6-10; Adams et al., 1990, *J Clin Microbiol* 28:998-1001; Adams et al., 1990, *J Leukoc Biol* 48:549-56; Tsai et al., 1992, *Cell Immunol* 144:203-16; and Pfister et al., 1992, *Immunol* 77:473-6).

In this assay, adherent J774 cells were removed from culture, washed with PBS-1mM EDTA, and then washed twice with complete tissue culture medium without

antibiotics. 2×10^6 to 4×10^6 J774 cells/100 mm culture dish were incubated in tissue culture medium overnight in a CO₂ incubator. Adherent J774 cells are removed with PBS-1mM EDTA, washed three times in tissue culture medium, and adjusted to 5×10^5 /ml. Aliquots of 50 μ l were added per well of a round bottom 96 well plate. The plate is then incubated for 1 hour at 37°C in a CO₂ incubator. Per well is added either an *htrB* mutant, or the wild type strain, in various colony forming units (cfu, infection dose). The plate is then incubated at 37°C for 1 hour in a CO₂ incubator. After the incubation, 100 μ l of culture medium containing 50 μ g/ml gentamycin is added per well. The plate is then incubated overnight at 37°C in a CO₂ incubator. Aliquots of 50 μ l of the J774 supernatant were removed per well and transferred into wells of a flat bottom 96 well plate. Serial 10 fold dilutions were made of the J774 supernatant. Included as a control is a dilution series of recombinant TNF α (rTNF α). Added per well is 50 μ l of WEHI 164 clone 13 cells at 6×10^5 cells/ml in tissue culture medium + 25 mM LiCl + 2 μ g/ml actinomycin D; and the mixture was incubated overnight at 37°C in a CO₂ incubator. After the incubation, 10 μ l of alomar blue is added, and 5-7 hours later the optical density is read at 570 nm. The assay utilizes alomar blue as a color indicator; i.e., alomar blue is converted to a red color by living cells, but remains blue if the cells are killed.

FIG. 3 shows a comparison between the number of bacterial cells of *H. influenzae* strain 2019 (wild type, \square), and of bacterial cells of *htrB* mutant NTHi B29 (O and Δ) necessary to stimulate the release of enough TNF α from J774 cells to kill the TNF α -susceptible cell line WEHI 164. B29_H (Δ) and B29_{Lo} (O) refer to a high number (>3) and low number (<3) of passages of *htrB*

mutant, respectively. As shown in FIG. 3, the *htrB* mutant shows a reduced ability to stimulate $\text{TNF}\alpha$ release; i.e., between an approximately 10 fold reduction (B29_{Lo}) to an approximately 100 fold reduction (B29_{Hi}). This reduced ability to stimulate $\text{TNF}\alpha$ is one indication of the *htrB* mutant being substantially reduced in toxicity due to the lack of one or more secondary acyl chains in the lipid A portion of the endotoxin.

The effect due to the lack of one or more secondary acyl chains on the toxicity of a gram-negative bacterial pathogen was also examined using a standard *in situ* assay for measuring *in vivo* toxicity. SV-40 transformed human respiratory epithelial cells and human primary respiratory epithelial cells, when stimulated by endotoxin, produces $\text{TNF}\alpha$ which production can be demonstrated by detection of $\text{TNF}\alpha$ mRNA using methods known to those skilled in the art for *in situ* hybridization (a modification of MacNaul et al., 1990, *J. Immunol.* 145:4154-66). The cells are grown in a monolayer within wells of a 24-well plate until approximately confluent. To stimulate the cells, $1\mu\text{g/ml}$ of LOS is added, and the cells are incubated overnight at 37°C . A counterstain (e.g., membrane stain) is added and the cells are then fixed with 0.5 ml of 2% paraformaldehyde in buffer so that the counterstain is fixed directly into the cells. Hybridization is then performed using an oligonucleotide probe specific for $\text{TNF}\alpha$ RNA (SEQ ID NO:4), and a control probe (SEQ ID NO:5). The amount of $\text{TNF}\alpha$ mRNA visually detected is directly proportional to the toxicity of the stimulating LPS. $\text{TNF}\alpha$ mRNA was minimally induced in SV-40 transformed human respiratory epithelial cells, and human primary respiratory epithelial cells (FIG. 4), exposed to LOS isolated from the *htrB* mutant NTHi B29. This is in contrast to LOS isolated from parent strain NTHi 2019 which induced high

levels of TNF α mRNA in these human respiratory cells (FIG. 4) and cell lines.

The substantial reduction in toxicity exhibited by the *htrB* mutant, as observed by the TNF α assays, due to the lack of one or more secondary acyl chains is further supported by previously reported assays of bioactivity of endotoxin treated with acyloxyacyl hydrolase which selectively removes the secondary acyl chains from endotoxin. Deacylated endotoxin from *E. coli*, *H. influenzae*, *N. meningitidis*, and *S. typhimurium* were (a) similarly reduced in potency in the Limulus lysate test relative to the respective wild type endotoxin; (b) reduced in the ability to stimulate neutrophil adherence to human endothelial cells relative to the respective wild type endotoxin; and (c) reduced in mitogenic activity for murine splenocytes (Erwin et al., 1991, *Infect Immun* 59:1881-1887); yet maintained expression of antigenic epitopes. Similarly, *S. typhimurium* LPS treated with acyloxacyl hydrolase showed a reduction in toxicity by 100-fold or greater in a dermal Shwartzman reaction; was less pyrogenic in a thermal response model; showed a 5 to 12 fold reduction in B-cell mitogenicity; and showed a 10 to 20 fold reduction in the release of prostaglandin E₂, as compared to wild type endotoxin, in concluding that maximally deacylated LPS was at least 10 fold less toxic than wild type endotoxin (U.S. Patent No. 4,929,604).

EXAMPLE 4

30 Use of *htrB* mutants as immunogens

In one aspect of this embodiment, the *htrB* mutant of a gram-negative bacterial pathogen is used as a whole cell vaccine. The benefit of using live, attenuated (weakened in its ability to cause pathogenesis) bacteria as an immunogen in a vaccine formula is that they are able to survive and may persist

in the human or animal body, and thus confer prolonged immunity against disease. In conjunction with the benefit of using a live bacteria to prolong the immune response, gram-negative bacterial pathogen *htrB* mutants have the added benefit in that they exhibit substantially reduced toxicity. Another advantage, as compared to a vaccine formulation comprising an isolated peptide representing a bacterial antigen, is that a bacterial antigen expressed on the surface of a bacterial cell will often result in greater stimulation of the immune response. This is because the surface of bacteria of the family *Enterobacteriaceae* acts as a natural adjuvant to enhance the immune response to an antigen presented thereon (Wezler, 1994, *Ann NY Acad Sci* 730:367-370). Thus, using a live bacterial vaccine, such as an *htrB* mutant, to express complete proteins in an native conformation (i.e., as part of the bacterial outer membrane) is likely to elicit more of a protective immune response than an isolated protein alone.

Live bacterial vaccine vectors of the family *Enterobacteriaceae* that have been described previously include attenuated *Salmonella* strains (Stocker et al., U.S. Patent Nos. 5,210,035; 4,837,151; and 4,735,801; and Curtiss et al., 1988, *Vaccine* 6:155-160; herein incorporated by reference), and *Shigella flexneri* (Sizemore et al., 1995, *Science* 270:299-302; herein incorporated by reference). One preferred embodiment is to provide a vaccine delivery system for human or animal (depending on the genus and species of the gram-negative bacterial pathogen) mucosal pathogens. Thus, immunization by the parental route or by the mucosal route with a prophylactically effective amount of the *htrB* mutant, or an *htrB* mutant transformed to recombinantly express additional bacterial antigens (that do not negatively affect the growth or replication of the transformed *htrB* mutant), can lead to

colonization of mucosal surfaces to induce mucosal immunity against the antigens displayed on the surface of, or secreted from the *htrB* mutant. The resultant *htrB* mutant can be used in a vaccine formulation which
5 expresses the bacterial antigen(s).

Similar methods can be used to construct an inactivated *htrB* mutant vaccine formulation except that the *htrB* mutant is inactivated, such as by chemical means known in the art, prior to use as an immunogen and
10 without substantially affecting the immunogenicity of the expressed immunogen(s). For example, human bronchial mucosal immunity has been stimulated with an aerosol vaccine comprising lysed *H. influenzae* (Latil et al., 1986, *J Clin Microbiol* 23:1015-1021). Either of
15 the live *htrB* mutant vaccine or the inactivated *htrB* mutant vaccine may also be formulated with a suitable adjuvant in order to further enhance the immunological response to the antigen(s) expressed by the vaccine vector, as to be described in more detail.

20 In another aspect of this embodiment, the endotoxin is isolated from the *htrB* mutant using methods known in the art, and the isolated *htrB* endotoxin is used in a vaccine formulation. As mentioned previously, major antigenic determinants of gram-negative bacteria
25 are believed to reside in the carbohydrate structure of the O-specific side chain of LPS and the complex carbohydrate structure of LOS. However, the chemical nature of LPS and LOS prevent the use of these molecules in vaccine formulations; i.e., active immunization with
30 LPS or LOS is unacceptable due to the inherent toxicity of the secondary acyl chains of the lipid A portion of endotoxin. The endotoxin isolated from an *htrB* mutant of a gram-negative bacterial pathogen lacks one or more
secondary acyl chains, and thus exhibits substantially
35 reduced toxicity as compared to endotoxin isolated from the respective wild type bacteria. Therefore, endotoxin

isolated from an *htrB* mutant of a gram-negative bacterial pathogen can be used in a vaccine formulation in inducing immunity against the respective wild type gram-negative bacterial pathogen. LPS or LOS can be isolated by the phenol-water method (Westphal et al., 1965, *Methods in Carbohydrate Chemistry* 5:83-91); or using an alternative purification procedure (using a protease; Hitchcock et al., 1983, *J. Bacteriol.* 154:269-277).

Many methods are known for the introduction of a vaccine formulation into the human or animal (collectively referred to as "individual") to be vaccinated. These include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, ocular, intranasal, and oral administration. Conventionally, vaccine formulations containing either live bacteria, or attenuated or inactivated bacteria, are administered by injection or by oral administration. For example, respiratory immunity can be stimulated by intestinal immunization with purified *H. influenzae* antigens (Cripps et al., 1992, *J. Infect Dis* 165S1:S199-201; herein incorporated by reference). The vaccine formulation may comprise a physiologically acceptable solution as a carrier in which the *htrB* mutant bacterial cells or isolated *htrB* mutant endotoxin is suspended. Various adjuvants may be used in conjunction with vaccine formulations. The adjuvants aid by modulating the immune response and in attaining a more durable and higher level of immunity using smaller amounts of vaccine antigen or fewer doses than if the vaccine antigen were administered alone. Examples of adjuvants include incomplete Freund's adjuvant, Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate), oil emulsions, Ribi adjuvant, the pluronic polyols, polyamines, Avridine, Quil A, saponin, MPL, QS-21, and mineral gels

such as aluminum hydroxide, aluminum phosphate, etc. The vaccine formulation is administered in a prophylactically effective amount to be immunogenic, which depends on factors including the individual's ability to mount an immune response, the degree of protection to be induced, and the route of administration.

In another aspect of the invention, the vaccine formulation can be administered orally by including it as part of the feed given to economically important livestock. As known by those skilled in the art, species of *Haemophilus*, *Campylobacter*, *Pseudomonas*, and *Salmonella* are pathogenic for economically important livestock. Using the methods according to the present invention, as illustrated in the following examples, *htrB* mutants of such animal pathogens can be produced. The resultant *htrB* mutants, or endotoxin isolated therefrom, can be used in a vaccine formulation. Use of vaccine formulations, containing one or more antigens of various microbial pathogens, in animal feed has been described previously (See for example, Pritchard et al., 1978, *Avian Dis.* 22:562-575).

EXAMPLE 5

H. influenzae htrB mutants as immunogens

In one embodiment, the *htrB* mutant is an *H. influenzae htrB* mutant. *Haemophilus influenzae* is an important human respiratory tract pathogen in diseases including otitis media, chronic sinusitis, and chronic obstructive pulmonary disease. Certain surface-exposed bacterial components, including P2, P6, and LOS, appear to be antigens which may confer a protective immune response in immunized humans. Such antigens have been shown to be targets of bactericidal antibody, and the presence of serum bactericidal antibody is associated

with protection from infection by *H. influenzae* (Faden et al., 1989, *J. Infect. Dis.* 160:999-1004).

5.1 In one aspect of the this embodiment, the endotoxin isolated from an *htrB* mutant is used as the immunogen in a vaccine formulation. As demonstrated in Example 3 herein, the endotoxin isolated from an *htrB* mutant of a gram-negative bacterial pathogen lacks one or more secondary acyl chains, and thus exhibits substantially reduced toxicity as compared to endotoxin isolated from the respective wild type bacterial pathogen. Therefore, endotoxin isolated from an *htrB* mutant of *H. influenzae* can be used in a vaccine formulation in inducing immunity against the respective wild type strain. The *htrB* mutant LOS may be isolated by a method known to those skilled in the art for isolating LOS. The *htrB* mutant LOS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

To illustrate the effects of immunization with *htrB* mutant LOS, a mouse model was used. NTHi 2019 LOS, and *htrB* mutant NTHi B29 LOS were each isolated from their respective strains using the phenol-water method. Groups of at least 5 Swiss Webster mice were immunized subcutaneously with 1 μ g of either NTHi 2019, *htrB* mutant B29 LOS, or *htrB* mutant conjugated to a carrier protein, with adjuvant QS-21. Sera was collected from each group 5 weeks after immunization, and the sera from animals comprising a group were pooled. The pooled sera was assessed for titer of anti-LOS antibody by enzyme-linked immunosorbent assay (ELISA). Microtiter wells of ELISA plates were coated with either 10 μ g of NTHi 2019, or *htrB* mutant B29 LOS. FIG. 5 illustrates the mean titers of anti-LOS antibody against NTHi 2019 LOS (antigen coating) in ELISA from mice immunized with NTHi

2019 (Pool 595), *htrB* mutant B29 LOS (Pool 597), or *htrB* mutant B29 LOS conjugated to a carrier protein (Pool 606). FIG. 6 illustrates the mean titers of anti-LOS antibody against *htrB* mutant B29 LOS (antigen coating)
 5 in ELISA from mice immunized with NTHi 2019 (Pool 595), *htrB* mutant B29 LOS (Pool 597), or *htrB* mutant B29 LOS conjugated to a carrier protein (Pool 606). Pre-immune sera was also included as a control ("week 0" or "time 0").

10 The antibody induced by the different LOS preparations were then tested for functional activity by performing bactericidal assays using NTHi 2019 as the target. Into the wells of a 96-well plate are added
 15 0.150 ml buffer, 0.040 ml pooled human sera as a complement source, and 0.01ml of NTHi 2019. Typically, the organism is plated in a dilution (e.g. 20 to 200 cfu). Into test wells are added the respective antisera either undiluted ("neat"), 1/10 dilution, or 1/100
 20 dilution. The plate is then rotated vigorously (175-200 rpm) at 37°C for 30 minutes. Aliquots from respective wells are plated on media and grown to determine percentage survival, and log kill. Log kill is
 25 calculated as the log(cfu 30 minutes/cfu time 0). The results of the bactericidal assay are shown in Table 1, where Group R595 is the antisera induced by NTHi 2019
 LOS, Group R597 is the antisera induced by *htrB* mutant B29 LOS, and Group R606 is the antisera induced by *htrB* mutant B29 LOS conjugated to a carrier protein.

Table 1

Group	week	dilution	% survival	log kill
R595	0	neat	1.00	-1.99
		1/10	37.30	-0.43
		1/100	87.20	-0.06
	5	neat	-	-4.49
		1/10	0.07	-3.15

		1/100	20.20	-0.70
	7	1/10	-	-4.62
		1/100	7.80	-1.11
R597	0	neat	3.50	-1.46
		1/10	62.40	-0.20
		1/100	108.60	0.04
	5	neat	-	-4.49
		1/10	4.40	-1.36
		1/100	82.40	-0.08
	7	1/10	-	-4.49
		1/100	99.80	0.00
R606	0	neat	0.13/110.5	-2.87/0.92
		1/10	130.20	0.11
		1/100	106.50	0.03
	5	neat	1.5/0.1	-1.81/-2.9
		1/10	25.10	-0.60
		1/100	164.10	0.22
	7	1/10	0.04	-3.44
		1/100	185.90	0.27

20
 25
 30

The greater the log kill (the more negative the number, e.g. -4.49), the greater the bactericidal activity is of the respective antibody. Thus, for comparison purposes, at week 7 and at a 1/10 dilution, log kill for the antisera induced by NTHi 2019 LOS is -4.62, the log kill for the antisera induced by NTHi *htrB* mutant B29 LOS is -4.49, and the log kill of the antisera induced by *htrB* mutant B29 LOS conjugated to a carrier protein is -3.44. By ELISA, it looks like the antisera induced by *htrB* mutant B29 LOS antibody is low in titer; yet, as demonstrated by the bactericidal assays, significant functional antibody is raised by immunization with *htrB* mutant B29 LOS.

5.2 In another aspect of the this embodiment, *htrB* mutant bacterial cells are used as the immunogen in a vaccine formulation. To illustrate the effects of immunization with *htrB* mutant bacterial cells, an infant rat model was used. The use of the infant rat model as a model of bacteremic infections due to type b *H. influenzae* (Hib) in humans, and for determining the virulence of type b *H. influenzae* strains, has been accepted by those skilled in the art (see, e.g., Smith et al., 1973, *Infect. Immun.* 8:278-290; Moxon et al., 1974, *J. Infect. Dis.* 129:154-62; Rubin et al., 1983, *Infect. Immun.* 41:280-284; Zwahlen et al., 1985, *J. Infect. Dis.* 152:485-492).

Type b strain A2 of *H. influenzae* has already been characterized as a highly virulent strain in the infant rat model system that causes bacteremia and meningitis after inoculation (e.g., intraperitoneal or intranasal), and as a clinical isolate from humans (it isolated from a child with meningitis due to *H. influenzae*). Using the methods according to Example 1, an *htrB* mutant was made from Hib strain A2. One week old infant Sprague-Dawley albino rats were inoculated intraperitoneally with either 10^7 Hib strain A2 or 10^7 *htrB* A2 mutant and then assessed for intravascular clearance by measuring the number of colony forming units (cfus) per ml of blood obtained 48 hours post-inoculation. The results showed that 20 of 20 infant rats inoculated with Hib strain A2 showed bacteremia, with all rats showing greater than 10^5 cfu/ml of strain A2. In contrast, only 13 of 20 infant rats inoculated with *htrB* A2 mutant showed bacteremia, with only 10 of the 13 showing greater than 10^5 cfu/ml of *htrB* A2 mutant. Similarly, one week old infant Sprague-Dawley albino rats were inoculated intranasally with either 10^7 Hib strain A2 or 10^7 *htrB* A2 mutant and then assessed for intravascular clearance. The results showed that 8 of

20 infant rats inoculated with Hib strain A2 showed bacteremia, with 7 of those 8 rats showing greater than 10^5 cfu/ml of strain A2. In contrast, none of the 30 infant rats inoculated with *htrB* A2 mutant showed bacteremia. Taken together, it can be concluded from this model system that *htrB* mutants demonstrate attenuated virulence, as compared to its wild-type strain, as indicated by the decreased ability to cause bacteremia (e.g., a 30% reduction in the occurrence of bacteremia).

To further illustrate the effects of immunization with *htrB* mutant bacterial cells, a chinchilla model was used. The use of the chinchilla model as a model of middle ear infections due to nontypable *H. influenzae* (NTHi) in humans, and for determining the virulence of NTHi strains, has been accepted by those skilled in the art (see, e.g., Bakaletz et al., 1989, *Acta Otolaryngol.* 107:235-243; Madore et al., 1990, *Pediatrics* 86:527-34; Barenkamp, 1986, *Infect. Immun.* 52:572-78; Green et al., 1994, *Methods Enzymol.* 235:59-68).

NTHi 2019 is a clinical isolate described previously (see, e.g., Murphy et al., 1986, *Infect. Immun.* 54:774-779). Each healthy adult chinchilla was inoculated, via the epitympanic bulla into the middle ear space, with various log doses of either NTHi 2019 or *htrB* mutant NTHi B29. The course of middle ear disease was then assessed by periodic otoscopic examination for tympanic membrane inflammation or middle ear infusion, and aspiration from the middle ear with subsequent culture. The results showed that when compared to NTHi 2019, it takes up to a 3 log greater dose of *htrB* mutant NTHi B29 (10^7 cfu/ear) to induce middle ear infection. It can be concluded from this model system that *htrB* mutants demonstrate attenuated virulence, as compared to

its wild-type strain, as indicated by the decreased ability to cause middle ear disease.

In another aspect of this embodiment the *H. influenzae* *htrB* mutant is genetically engineered to express one or more heterologous bacterial antigens. As will be discussed in more detail below, *H. influenzae* has a natural genetic transformation process involving linearized DNA binding, uptake via one or more uptake sequences (e.g. AAGTGCAGGT -SEQ ID NO:3), translocation, and recombination. Thus, one mechanism to introduce a recombinant DNA molecule containing the at least one heterologous bacterial antigen to be expressed, is to transform the host *H. influenzae* *htrB* mutant with linearized recombinant DNA molecule containing the DNA encoding the at least one heterologous bacterial antigen ("the encoding sequence"). Alternatively, the recombinant DNA molecule containing the encoding sequence to be expressed can be inserted into a plasmid vector, and either introduced into as a linearized recombinant molecule by the natural transformation process; as circularized recombinant plasmid using electroporation of noncompetent *H. influenzae* *htrB* mutants; or as a circularized recombinant plasmid transformed into competent *H. influenzae* *htrB* mutants.

Plasmids useful for cloning of and expression from recombinant DNA molecules in *H. influenzae* are known to those skilled in the art. Such plasmids include:

pRSF0885 confers ampicillin resistance, and contains a PvuII cloning site and a defective TnA sequence (Setlow et al., 1981, *J. Bacteriol.* 148:804-811), and can replicate in both *H. influenzae* and *E. coli* (Trieu et al., 1990, *Gene* 86:99-102).

pDM2 was constructed by cloning chloramphenicol resistance into pRSF0885; and pDM5 was constructed by

cloning tetracycline resistance into pRSF0885 (McCarthy et al., 1986, *J. Bacteriol.* 168:186-191).

pVT63, pVT64, pVT65, pVT66 are improved shuttle vectors for *H. influenzae* and *E. coli* based on pDM2 (Trieu et al., 1990, *Gene* 86:99-102), and contain the pUC-derivative of the ColE1 ori, and the pRSF0885 rep locus. Additionally, each plasmid has drug markers with unique restriction sites for insertional inactivation of the drug marker as follows: pVT63- ApR (*HincII*, *PstI*, *ScaI*), KmR (*ClaI*, *HindIII*, *NruI*, *SmaI*, *XhoI*); pVT64- ApR (*HincII*, *PstI*, *ScaI*, *SspI*), SpR; pVT65- ApR (*HincII*, *PstI*, *ScaI*, *PvuI*, *SspI*), CmR (*BalI*, *NcoI*); pVT66- ApR (*HincII*, *PstI*, *ScaI*, *PvuI*), CmR (*SmaI*).

pACYC177, pACYC184, pSU2718, pSU2719 are improved shuttle vectors for *H. influenzae* and *E. coli* based on p15A (Chandler, 1991, *Plasmid* 25:221-224), have the p15A ori, and were compatible with a plasmid containing the RSF0885 origin of replication. Additionally, each plasmid has multiple cloning sites restriction sites and drug markers as follows: pACYC177- ApR, KmR (Accession No. X06402); pACYC184- CmR, TcR (Accession No. X06403); pSU2718- CmR and polycloning site from pUC18 (Accession No. M64731); and pSU2719- CmR and polycloning site from pUC19 (Accession No. M64732).

pQL1 is an improved shuttle vector for use in *H. influenzae* and *E. coli* containing both the pMB1 ori and p15a ori, KmR which is flanked by *H. influenzae* uptake sequences, a multiple cloning site containing a unique *BamHI* and *SmaI* restriction sites, and which is particularly suited for analyzing *H. influenzae* promoter strength in *H. influenzae* (Heidecker et al., 1994, *Gene* 150:141-144).

In cloning the recombinant DNA molecule containing the encoding sequence into a plasmid vector, one skilled in the art will appreciate that the choice of restriction enzymes for digesting both the

recombinant DNA molecule and the plasmid to result in compatible ends for ligation depends on the unique restriction enzyme sites at the ends of the recombinant DNA molecule, whether occurring naturally or engineered such as during enzymatic amplification; one or more unique restriction enzyme sites within the plasmid vector; whether insertion into the plasmid vector will assist in the selection process (See, e.g., pVT66); and whether a plasmid-derived promoter is used solely, or in addition to the promoter(s) of the encoding sequences, to drive expression from the recombinant DNA molecule. Selection and screening of transformed *H. influenzae* *htrB* mutants may be accomplished by methods known in the art including detecting the expression of a marker gene (e.g., drug resistance marker) present in the plasmid, and immunodetection of the expressed and displayed heterologous bacterial antigen. While this aspect of the embodiment illustrates that the recombinant DNA molecule containing the encoding sequence can be inserted into a plasmid and expressed in *H. influenzae* *htrB* mutants, it will be appreciated by those skilled in the art that vectors other than plasmids, can be used including, but not limited to, bacteriophage vectors.

Successful expression of the at least one heterologous bacterial antigen requires that either the recombinant DNA molecule comprising the encoding sequence, or the vector itself, contain the necessary control elements for transcription and translation which is compatible with, and recognized by the particular host system used for expression. Using methods known in the art of molecular biology, including methods described above, various promoters and enhancers can be incorporated into the vector or the recombinant DNA molecule containing the encoding sequence to increase the expression of the heterologous bacterial antigen, provided that the increased expression of the

heterologous bacterial antigen(s) is compatible with (for example, non-toxic to) the *htrB* mutant. As referred to herein, the encoding sequence can contain DNA encoding more than one heterologous bacterial antigen, and may include viral and/or fungal antigen-encoding sequences, to create a multivalent antigen for use as an improved vaccine composition.

The selection of the promoter will depend on the expression system used. For example, a preferred promoter in an *H. influenzae* expression system may be the P2 or P6 promoter operatively linked to the encoding sequence. Promoters vary in strength, i.e. ability to facilitate transcription. Generally, for the purpose of expressing a cloned gene, it is desirable to use a strong promoter in order to obtain a high level of transcription of the gene and expression into gene product. For example, bacterial, phage, or plasmid promoters known in the art from which a high level of transcription has been observed in a host cell system comprising *E. coli* include the *lac* promoter, *trp* promoter, *recA* promoter, ribosomal RNA promoter, the *P_R* and *P_L* promoters, *lacUV5*, *ompF*, *bla*, *lpp*, and the like, and may be used to provide transcription of the inserted encoding sequence.

Other control elements for efficient gene transcription or message translation include enhancers, and regulatory signals. Enhancer sequences are DNA elements that appear to increase transcriptional efficiency in a manner relatively independent of their position and orientation with respect to a nearby gene. Thus, depending on the host cell expression vector system used, an enhancer may be placed either upstream or downstream from the encoding sequence to increase transcriptional efficiency. These or other regulatory sites, such as transcription or translation initiation signals, can be used to regulate the expression of the

encoding sequence. Such regulatory elements may be inserted into the recombinant DNA molecule containing the encoding sequence, or nearby vector DNA sequences using recombinant DNA methods described herein, and
5 known to those skilled in the art, for insertion of DNA sequences.

Accordingly, a recombinant DNA molecule containing an encoding sequence, can be ligated into an expression vector at a specific site in relation to the
10 vector's promoter, control, and regulatory elements so that when the recombinant vector is introduced into the *htrB* mutant, the heterologous bacterial antigen can be expressed in the host cell. The recombinant vector is then introduced into the *htrB* mutant, and the
15 transformed *htrB* mutants are selected, and screened for those cells containing the recombinant vector. Selection and screening may be accomplished by methods known in the art, and depending on the vector and expression system used.

20 The introduction of a recombinant DNA molecule containing the encoding sequence (including an expression vector or plasmid containing the same) into *H. influenzae htrB* mutants can be accomplished in any one of three processes: a natural genetic transformation
25 process; transformation of competent bacterial cells; and electroporation of non-competent bacterial cells.

Natural transformation process

The natural genetic transformation process of
30 *H. influenzae* involves linearized DNA binding, uptake via one or more uptake sequences, translocation, and recombination. Thus, one mechanism to introduce a recombinant DNA molecule containing the encoding sequence to be expressed into at least one heterologous
35 bacterial antigen, is to transform the host *H. influenzae* with linearized recombinant DNA molecule

containing the encoding sequence; or a linearized vector having inserted into it the recombinant DNA molecule containing the encoding sequence to be expressed. In this natural process, when the linearized DNA is translocated intracellularly, one of the translocated strands of DNA is apparently degraded by exonuclease activity (Barany et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:7274-7278). If the translocated strand lacks homology sufficient for recombination into the *H. influenzae* chromosome, the translocated strand becomes susceptible to further degradation (Pifer et al., 1985, *Proc. Natl. Acad. Sci. USA* 82:3731-3735). Using methods known in the art (e.g., Barany et al., 1983, *supra*; herein incorporated by reference), linearized DNA containing the encoding sequence can be introduced into *H. influenzae htrB* mutants. Since the encoding sequence can be flanked by *H. influenzae* sequences, to increase the likelihood of recombination of the encoding sequence into the *H. influenzae htrB* mutants' genome is likely to occur.

Transformation of competent bacterial cells

Another mechanism to introduce a recombinant DNA molecule containing the encoding sequence to be expressed into at least one heterologous bacterial antigen, is to transform competent host *H. influenzae htrB* mutants with a circular vector, such as a plasmid, having inserted into it the recombinant DNA molecule containing the encoding sequence to be expressed. Competence of *H. influenzae* develops best under conditions in which the bacterial cell duplication is inhibited, such as a temporary shift to anaerobic conditions, by physiological change occurring during late-log phase growth, and transfer of cells into nutrient-poor, chemically-defined medium. Such defined media for the development of competence of *H. influenzae*

has been previously described in detail (Herriott et al., 1970, *J. Bacteriol.* 101:517-524; herein incorporated by reference). It appears that only a short time after entering competent *H. influenzae*, a plasmid containing sequences homologous to the bacterial chromosome can insert its homologous sequence (such as the encoding sequence flanked by *H. influenzae* sequences) into the chromosome via recombination (Setlow et al., 1981, *supra*). for expression. Thus, in this embodiment, a plasmid containing the encoding sequence which is capable of being transformed into competent *H. influenzae htrB* mutants is introduced by methods for transformation known in the art (Karudapuram et al., 1995, *J. Bacteriol.* 177:3235-3240; Setlow et al., 1981, *supra*, herein incorporated by reference). The encoding sequence may then be recombined into the *H. influenzae htrB* mutants' genome where it is expressed under the control of its own promoter or an *H. influenzae* promoter near the site of insertion. Such transformation is reported to be at a relatively high frequency (McCarthy and Cox, 1986, *J. Bacteriol.*, 168:186-191).

Alternatively, transformation of competent *H. influenzae htrB* mutants by a circular plasmid with the appropriate origin(s) of replication and containing the encoding sequence may result in plasmid establishment; i.e., a plasmid coexisting as an extrachromosomal element without recombination. Examples of such plasmids have been described above. Thus, in this variation of the embodiment, a plasmid containing the encoding sequence which is capable of being transformed into, and established in, competent *H. influenzae htrB* mutants is introduced by methods for transformation known in the art. The encoding sequence is then expressed from the plasmid under the control of its own promoter or a promoter within the vector.

Electroporation of non-competent bacterial cells

Yet another mechanism to introduce a recombinant DNA molecule containing the encoding sequence to be expressed into at least one heterologous bacterial antigens, is to introduce a circular vector, such as a plasmid having inserted into it the recombinant DNA molecule containing the encoding sequence to be expressed, into non-competent host *H. influenzae htrB* mutants by electroporation.

Electroporation has been used to efficiently introduce plasmid DNA into bacteria. However, optimal conditions may differ depending on the host cell used. Optimal conditions have been described for electroporating plasmid DNA into *H. influenzae* (Mitchell et al., 1991, *Nucl. Acids Res.* 19:3625-3628; herein incorporated by reference). It was found that electroporation of plasmid into *H. influenzae* made competent by defined, nutrient poor media was several orders of magnitude less efficient than electroporation into non-competent *H. influenzae*. Thus, in this variation of the embodiment, it would be preferred that a plasmid containing the encoding sequence is electroporated into non-competent *H. influenzae htrB* mutants. The plasmid is capable of being established in *H. influenzae htrB* mutants, or is degraded after the encoding sequence has recombined into the *H. influenzae htrB* mutants' genome. In either case, the encoding sequence is under the control of its own promoter; or a promoter within the vector or genome, respectively.

EXAMPLE 6

Neisserial htrB mutants as immunogens

In another embodiment, the *htrB* mutant is a *Neisserial htrB* mutant selected from the group including *Neisseria gonorrhoeae*, and *Neisseria meningitidis*. *N. gonorrhoeae* is a gram-negative bacterial pathogen

causing the sexually transmitted disease gonorrhea, which subsequently can lead to pelvic inflammatory disease in females. *N. meningitidis* is a gram-negative bacterial pathogen which can cause a variety of clinical
5 infections including bacteremia, septicemia, meningitis, and pneumonia. Alterations in the terminal glycosylation of the LOS of *Neisseria* are believed correlate with serum sensitivity and serum resistance of the organism. Further, protective bactericidal antibody
10 is directed against type-specific antigens of *N. meningitidis*, wherein the type-specific antigens have been identified as outer membrane proteins, or LOS, or both.

Using the methods according to the present
15 invention, as illustrated in Examples 1-3 and 10, *htrB* mutants of a *Neisserial* species can be produced and identified. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of the *Neisserial* species, and produce a mutated *htrB* gene
20 (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in a *Neisserial htrB* mutant lacking one or more secondary acyl chains. Alternatively, there may be sufficient
homology between *Neisseria* and *Haemophilus* to use
25 plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform the *Neisserial* species for recombination of the mutant *htrB* gene into the
30 *Neisserial htrB* gene. *Neisserial* transformants are then selected for by growth in the presence of chloramphenicol (1.5 µg/ml), resulting in identification of *Neisserial htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *Neisserial htrB*
35 mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences.

The resultant *Neisserial htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

5 Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *Neisserial htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *Neisserial* pathogens. The *htrB*
10 mutant LOS may be isolated by a method known to those skilled in the art for isolating LOS. The *htrB* mutant LOS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a
15 physiological solution), an adjuvant, or a carrier protein.

 Alternatively, *Neisserial htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be
20 genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant
DNA molecules into *Neisserial* species are known to those
25 skilled in the art, including: *pLES2* confers ampicillin resistance, is a shuttle vector functional in both *E. coli* and *N. gonorrhoeae*, and contains a polylinker with restriction sites for *EcoRI*, *SmaI*, and *BamHI* (Stein et al., 1983, *Gene* 25:241-247).
30 *Neisserial* species also contain a natural transformation process (Rudel et al., 1995, *Proc Natl Acad Sci USA* 92:7896-90; Goodman et al., 1991, *J Bacteriol* 173:5921-5923); and can also be made competent or be
electroporated using techniques known to those skilled
35 in the art.

EXAMPLE 7

Haemophilus ducreyi htrB mutants as immunogens

In another embodiment, the mutant is a *H. ducreyi htrB* mutant. *H. ducreyi* is a gram-negative bacterial pathogen causing a genital ulcer disease, chancroid. Using the methods according to the present invention, as illustrated in Examples 1-3 and 10, *H. ducreyi htrB* mutants can be produced and identified. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *H. ducreyi*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in an *H. ducreyi htrB* mutant lacking one or more secondary acyl chains. Alternatively, there is likely sufficient homology between *H. ducreyi* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *H. ducreyi* for recombination of the mutant *htrB* gene into the *H. ducreyi htrB* gene. *H. ducreyi* transformants are then selected for by growth in the presence of chloramphenicol (1.5 µg/ml), resulting in identification of *H. ducreyi htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *H. ducreyi htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *H. ducreyi htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin. Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from an *H. ducreyi htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *H. ducreyi*. The *htrB* mutant LOS

may be isolated by a method known to those skilled in the art for isolating LOS. The *htrB* mutant LOS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a
5 pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *H. ducreyi htrB* mutants can be used in a live bacterial vaccine preparation, in an
10 inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant
15 DNA molecules into *Haemophilus* species are known to those skilled in the art, as disclosed in Example 5; and can also be made competent or be electroporated using techniques known to those skilled in the art, as disclosed in Example 5.

20

EXAMPLE 8

Campylobacter jejuni htrB mutants as immunogens

In another embodiment, the mutant is a *C. jejuni htrB* mutant. *Campylobacter jejuni* is a gram-
25 negative bacterial pathogen causing human enteritis. Infection by *C. jejuni* has also been associated with the onset of neurologic disorders such as Guillian-Barré syndrome. *C. jejuni htrB* mutants can be produced and identified using the methods according to the present
30 invention, as illustrated in Examples 1-3 and 10. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *C. jejuni*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent
35 recombination resulting in an *C. jejuni htrB* mutant lacking one or more secondary acyl chains.

Alternatively, there may be sufficient homology between *C. jejuni* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *C. jejuni* for recombination of the mutant *htrB* gene into the *C. jejuni htrB* gene. *C. jejuni* transformants are then selected for by growth in the presence of chloramphenicol (1.5 μ g/ml), resulting in identification of *C. jejuni htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *C. jejuni htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *C. jejuni htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *C. jejuni htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *C. jejuni*. The *htrB* mutant LPS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant LPS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *C. jejuni htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant

DNA molecules into *C. jejuni* are known to those skilled in the art, and includes:

pUA466 confers tetracycline resistance, and contains an unique *Ava*I site and *Ava*II site (Taylor, 1986, *J Bacteriol* 165:1037-39).

C. jejuni can also be made competent or be electroporated using techniques known to those skilled in the art.

10

EXAMPLE 9

Moraxella catarrhalis htrB mutants as immunogens

In another embodiment, the mutant is a *M. catarrhalis htrB* mutant. *Moraxella catarrhalis* is a gram-negative bacterial pathogen causing otitis media in children; sinusitis and conjunctivitis in children and adults; and lower respiratory tract infections, septicemia, and meningitis in immunocompromised hosts. *M. catarrhalis htrB* mutants can be produced and identified using the methods according to the present invention, as illustrated in Examples 1-3 and 10. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *M. catarrhalis*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in an *M. catarrhalis htrB* mutant lacking one or more secondary acyl chains. Alternatively, there may be sufficient homology between *M. catarrhalis* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *M. catarrhalis* for recombination of the mutant *htrB* gene into the *M. catarrhalis htrB* gene. *M. catarrhalis* transformants are then selected for by growth in the presence of chloramphenicol (1.5 µg/ml), resulting in identification of *M. catarrhalis*

htrB mutant strains. Locations of the mTn3 insertion in the chromosomes of the *M. catarrhalis htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *M. catarrhalis htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *M. catarrhalis htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *M. catarrhalis*. The *htrB* mutant LOS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant LOS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *M. catarrhalis htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *M. catarrhalis* are known to those skilled in the art. *M. catarrhalis* contains a natural transformation process (Juni, 1977, *J Clin Microbiol* 5:227-35) and can also be made competent or be electroporated using techniques known to those skilled in the art.

EXAMPLE 10

Salmonella htrB mutants as immunogens

In another embodiment, the mutant is a *Salmonella htrB* mutant. *Salmonella* species comprise gram-negative bacteria that can cause a variety of clinical illnesses in humans and animals. For example, *S. typhi* is the causative agent of typhoid fever in humans. *S. paratyphi* is a causative organism of a fever known as salmonella fever in humans. Salmonellosis, a gastroenteritis in humans, can be caused by various species in the genus *Salmonella* (*typhimurium*, *newport*, *heidelberg*, and *enteritidis*). *Salmonella htrB* mutants can be produced and identified. One skilled in the art, using the *htrB* gene of a gram-negative bacterial pathogen, can produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis and subsequent recombination, ultimately resulting in an *Salmonella htrB* mutant having a modification in one or more secondary acyl chains. The resultant *Salmonella htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

To illustrate this embodiment, and using methods similar to those in Example 1 herein, mutagenesis of the *htrB* gene was carried out by shuttle mutagenesis by mini-Tn10 (conferring tetracycline resistance) used as an insertion sequence to mutate the *htrB* gene. The *htrB*:Tn10 was then transferred from *E. coli* to a virulent *S. typhimurium* by transduction. Using methods previously described (Masters, 1996, in *E. coli and Salmonella Cellular and Molecular Biology*, vol.2, 2nd edition, p. 2421, ASM Press), a *rm⁺ gale mutS recD* *S. typhimurium* (SL5283) was sequentially transduced with MST3488 (*recD542*:Tn10d which confers chloramphenicol resistance (*cm^r*)) via *Salmonella* phage P22 resulting in a *rm⁺ gale mutS recD cm^r* *S. typhimurium* ("MGS-1"), and then with MST3063 (*mutS*:Tn10 which

confers tetracycline resistant (tet^r) resulting in a r^m galE mutS recD cm^r tet^r *S. typhimurium* ("MGS-3"). *S. typhimurium* MGS-3 was cured of Tn10 by selection for tetracycline sensitivity on media using methods
5 previously described (Bochner et al., 1990, *J. Bacteriol.* 143:926-933) resulting in a r^m galE mutS recD cm^r *S. typhimurium* ("MGS-7"). For confirmation purposes, it was shown that *S. typhimurium* MGS-7 showed the same response to ultraviolet light as the parental
10 strain MGS-3.

An *E. coli* strain ("MLK217") containing the htrB:mini Tn10 was used to transfer the htrB:Tn10 by transduction into *S. typhimurium* MGS-7 via coliphage P1 according to methods previously described (Masters,
15 1996, *supra*), and selected for by growth at 30°C on media plates containing tetracycline. The result of the transduction, and after reisolation for tetracycline-resistant clones, was the creation of a r^m galE mutS recD htrB:mini Tn10 cm^r tet^r *S. typhimurium* ("MGS-23").
20 *S. typhimurium* MGS-23 was tested for one or more of the phenotypic properties associated with htrB mutation, namely (1) temperature sensitivity; (2) filamentation and bulging at non-permissive temperatures; and (3) deoxycholate resistance. The results of the phenotypic
25 analysis indicated that MGS-23 carried the miniTn10 element inserted within the *S. typhimurium* htrB gene because MGS-23 was able to grow at 30°C but not at 37°C; formed many filamentous forms when shifted to non-permissive temperature; and showed resistance to higher
30 concentrations of deoxycholate (7.5% to 10%) than the isogenic parent (2.5%). The mutation of the htrB was further confirmed by analysis using polymerase chain reaction.

A virulent *S. typhimurium* strain (SL1344) was
35 transduced to htrB:Tn10 from *S. typhimurium* MGS-23 via *Salmonella* phage P22 and selection at 30°C on media

plates containing tetracycline. After isolation, resultant tetracycline resistant clones having the same phenotype as MGS-23 were further analyzed. One such clone, MGS-31, was shown to have a mutated *htrB* gene, by
5 complementing the clone using a plasmid with a wild type *htrB* gene (Karow et al., 1991, *J. Bacteriol.* 173:741-50; Karow et al., 1991, *Mol. Microbiol.* 5:2285-2292) thereby returning the clone to the wild type phenotype of normal growth, normal cell morphology, deoxycholate sensitivity
10 at 37°C, and wild type virulence.

Endotoxin characteristics

Mass spectrometry was used to analyze the lipid A according to the methods in Example 2 herein. More specifically, lipid A from *S. typhimurium htrB*
15 mutant LPS and from wild type *S. typhimurium* LPS were each analyzed by liquid secondary ion mass spectrometry (LSIMS) in the negative ion mode to provide a spectrum of molecular ions for the different components lipid A. The chemical analysis of the lipid A of the *S.*
20 *typhimurium htrB* mutant indicated that the modifications in the lipid A structure that occurred were similar, but not identical, to modifications in the lipid A structure seen in *H. influenzae htrB* mutants. In the wild type *S.*
typhimurium lipid A contains either six (hexaacyl) or
25 seven (heptaacyl) fatty acid substitutions from the diglucosamine backbone (FIG. 7). In the wild type strain, on glucosamine II, the 3' substitution on the N-linked C14 fatty acid (hexaacyl or heptaacyl) is a C12 fatty acid. In contrast, and as shown in FIG. 7, the
30 C12 fatty acid is replaced with a C16 fatty acid. These results indicate that the *S. typhimurium htrB* gene encodes an acyltransferase responsible for placing the C12 fatty acid at the 3' position on the N-linked C14 fatty acid. Mutation of the *S. typhimurium htrB* gene
35 results in the functional induction of another acyltransferase which places a C16 fatty acid at the 3'

position on the N-linked C14 fatty acid. It is known to those skilled in the art, that lipid A is crucial for the survival of a gram-negative organism, and for the proper organization of its outer membrane. Thus, and as
5 related to virulence and toxicity of the organism, the effects of the *htrB* gene mutation in *S. typhimurium* was analyzed.

Endotoxin toxicity

A mouse model system that is used by those
10 skilled in the art as relevant to human disease, is the D-galactosamine model. In the D-galactosamine model, the sensitivity to LPS is increased by exposure to D-galactosamine (Galanos et al., 1986, *Infect. Immun.* 51:891-896) thereby achieving the same lethality and TNF
15 induction with low doses of LPS, i.e. with levels of endotoxin commensurate with those identified in the blood of septic patients. Thus, D-galactosamine-treated mice exposed to LPS is a standard animal model system accepted by those skilled in the art as relevant to
20 endotoxic shock in humans. In this model, groups of 4 mice were treated with D-galactosamine (8 mg) simultaneously with the administration of the dosage of the LPS to be tested. Groups of 4 mice each were injected with either 0.001 μ g, 0.01 μ g, 0.1 μ g, 1 μ g or
25 10 μ g of the purified LPS to be tested and the number of mice surviving the challenge at each dose were checked every 24 hours for 5 days after the injection. The LD₅₀ (lethal dose where 50% of the mice are killed) is then calculated. The purified LPS to be separately tested
30 included LPS from the wild type virulent *S. typhimurium* strain 1344; and the *S. typhimurium htrB* mutant (MGS-31). The results show that the LD₅₀ for mice injected with LPS from *S. typhimurium* strain 1344 is 0.01 μ g. In contrast, the LD₅₀ for mice injected with LPS from *S.*
35 *typhimurium htrB* mutant MGS-31 is 0.1 μ g. Thus, the lipid A from the *S. typhimurium htrB* mutant is at least

10 fold less toxic than the lipid A of the wild type strain.

Virulence

Since mice are naturally susceptible to infection by *S. typhimurium*, like humans, a second mouse model was used to assess the effects of the *htrB* mutation on virulence of *S. typhimurium*. Typically, from approximately 50 cfu to 100 cfu will kill 50 to 100% of the mice injected. The strains used included *S. typhimurium* strain 1344; *S. typhimurium htrB* mutant (MGS-31); and the *S. typhimurium htrB* mutant which was complemented by the plasmid containing an intact *htrB* gene (MGS-43). The respective organisms were injected intraperitoneally in a dosage ranging from 5×10^1 to 5×10^7 cfu and watched over 5 days. Generally, 100% of the animals who will die from bacteremia, do so within that period. As may be expected, the LD_{50} for the virulent *S. typhimurium* strain 1344 was less than 5×10^1 cfu. Likewise, the LD_{50} for the *S. typhimurium htrB* mutant which was complemented with the intact *htrB* gene, MGS-43, also was less than 5×10^1 cfu. In contrast, the LD_{50} for the *S. typhimurium htrB* mutant MGS-31 was 9.7×10^6 cfu, an approximately 2×10^5 reduction in virulence compared to the wild type virulent strain. *S. typhimurium htrB* mutant growth in vivo in mice was confirmed by culturing and assaying liver and spleen for bacterial counts. The results suggest that the *htrB* mutation in *Salmonella* has a more profound effect on virulence factors than just a modification of the LPS. Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *Salmonella htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of the *Salmonella* species. The *htrB* mutant LPS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant

LPS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

- Alternatively, *Salmonella htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *Salmonella* are known to those skilled in the art, and includes:
- 15 pYA260 containing *lacZ* cloned into a *trc* promoter; and pJW270 conferring tetracycline resistance and containing *lacI* (Ervin et al., 1993 *Microb Pathogen* 15:93-101).
 - pB7 confers kanamycin and chloramphenicol resistance, and contains a cloning site flanked by a *BalI* site and a
 - 20 *HindIII* site (Purcell et al., 1983, *Infect Immun* 39:1122-1127).
 - pACK5 contains the replicon of pAC1 from *Acetobacter pasteurianus* and confers kanamycin resistance (Grönes et al., 1995, *Biochem Biophys Res Commun* 206:942-947).
 - 25 pVAC468 is a suicide vector for chromosomal insertion of heterologous antigens into *Salmonella* and contains a polylinker having the following restriction sites: *ClaI*, *EcoRV*, *XhoI*, *SacI*, *SalI*, *SmaI*, *XbaI*, and *BglII* (Hohmann et al., 1995, *Proc Natl Acad Sci USA* 92:2904-2908).
 - 30 Also disclosed is a bacteriophage system, a 'chromosomal expression vector' for inserting genes encoding foreign antigens into the chromosome of *Salmonella*, which uses a defective transposable element carried on bacteriophage lambda (Flynn et al., 1990, *Mol Microb* 4:2111-2118).
 - 35 *Salmonella* can also be made competent (see for example, Purcell et al., 1983, *supra*) or be electroporated using

techniques known to those skilled in the art (see for example, Grones et al., 1995, *supra*; Coulson et al., 1994, *supra*).

EXAMPLE 11

5 *Shigella htrB* mutants as immunogens

In another embodiment, the mutant is a *Shigella* species *htrB* mutant. Members of the genus *Shigella* are gram-negative bacteria which cause diseases such as dysentery (pathogenic species include
10 *dysenteriae*, *sonnei*, and *flexneri*) primarily in humans. *Shigella htrB* mutants can be produced and identified using the methods according to the present invention, as illustrated in Examples 1-3 and 10. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate
15 the *htrB* gene of *Shigella*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in an *Shigella htrB* mutant lacking one or more secondary acyl chains. Alternatively, there may be sufficient
20 homology between *Shigella* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyl-transferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *Shigella* for
25 recombination of the mutant *htrB* gene into the *Shigella htrB* gene. *Shigella* transformants are then selected for by growth in the presence of chloramphenicol (1.5 μ g/ml), resulting in identification of *Shigella htrB* mutant strains. Locations of the mTn3 insertion in the
30 chromosomes of the *Shigella htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *Shigella htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled
35 in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from an *htrB* mutant made from a pathogenic *Shigella* species can be used in a vaccine formulation in inducing immunity against the wild type strains of *Shigella*. The *htrB* mutant LPS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant LPS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *Shigella htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *Shigella* are known to those skilled in the art, and includes: *PACK5* contains the replicon of *pAC1* from *Acetobacter pasteurianus* and confers kanamycin resistance. (Grones et al., 1995, *supra*). *Shigella* can also be made competent or be electroporated using techniques known to those skilled in the art.

EXAMPLE 12

Pseudomonas aeruginosa htrB mutants as immunogens

In another embodiment, the mutant is a *Pseudomonas aeruginosa htrB* mutant. *Pseudomonas aeruginosa* is a gram-negative bacterial pathogen which cause diseases such as respiratory tract infections and sepsis, particularly in immunocompromised patients. Other pathogenic species for humans and animals include *pseudomallei*, and *mallei*. Mass spectrometry and nuclear

magnetic resonance spectroscopy were used to determine the structure of lipid A of *Pseudomonas aeruginosa* LPS. The structure of *P. aeruginosa* lipid A was found to be the same as *Enterobacterial* lipid A: a backbone of a
5 glucosamine disaccharide which is either mono-phosphorylated or diphosphorylated (positions 1 and 4'); and which carries several molecules of ester- and amide-bound fatty acids. In addition to the hexaacyl and pentaacyl lipid A species, a tetraacyl species was
10 identified (Karunaratne et al., 1992, *Arch Biochem Biophys* 299:368-76).

Pseudomonas htrB mutants (e.g., *P. aeruginosa*) can be produced and identified using the methods according to the present invention, as illustrated in
15 Examples 1-3 and 10. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *Pseudomonas aeruginosa*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in a
20 *P. aeruginosa htrB* mutant lacking one or more secondary acyl chains. Alternatively, there may be sufficient homology between *P. aeruginosa* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT)
25 gene inserted into the *htrB* open reading frame at a different location, to transform *P. aeruginosa* for recombination of the mutant *htrB* gene into the *P. aeruginosa htrB* gene. *P. aeruginosa* transformants are then selected for by growth in the presence of
30 chloramphenicol (1.5 µg/ml), resulting in identification of *P. aeruginosa htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *P. aeruginosa htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences.
35 The resultant *P. aeruginosa htrB* mutants can then be tested for substantially reduced toxicity using assays

described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin
5 isolated from a *P. aeruginosa htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *P. aeruginosa*. The *htrB* mutant LPS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant LPS may be
10 used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

15 Alternatively, *P. aeruginosa htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine
20 preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *P. aeruginosa* are known to those skilled in the art, and includes:
pPAH121 confers carbenicillin resistance, and contains a
25 unique *HpaI* restriction site (Hoyne et al., 1992, *J Bacteriol* 174:7321-7327).

P. aeruginosa can also be made competent (see for example, Hoyne et al., 1992, *supra*) or be electroporated using techniques known to those skilled in the art.

30

EXAMPLE 13

Multivalent *htrB* mutant vaccine formulation

In one embodiment according to the present invention, as illustrated in Examples 4 & 5, the *htrB*
35 mutant is genetically engineered to express one or more heterologous microbial antigens in producing a

multivalent vaccine using methods known to those skilled in the art. In a preferred embodiment, a microbial pathogen may include a respiratory pathogen selected from the group of pathogens, with respective antigens, in Table 2.

Table 2

	PATHOGEN	INFECTION/DISEASE	PROTEIN ANTIGEN
	<i>H. influenzae</i>	otitis media, lower respiratory tract	D-15, P1, P6 ¹
10	Group A <i>Streptococcus</i>	pharyngitis, rheumatic fever	M ²
	<i>Branhamella catarrhalis</i>	otitis media, lower respiratory tract	CD, E ³
15	<i>Streptococcus pneumoniae</i>	pneumonia, otitis media, meningitis	autolysin, pneumolysin ⁴
	<i>Bordetella pertussis</i>	pertussis (whooping cough)	filamentous hemagglutinin, pertussis toxin, 69kDa Omp ⁵
	<i>Pseudomonas aeruginosa</i>	respiratory tract	Omp OprF, exotoxin A ⁶
20	<i>Legionella pneumophila</i>	pneumonia	OmpS, Hsp60 ⁷
	<i>Mycoplasma pneumoniae</i>	upper and lower respiratory tract	P1 ⁸
25	Respiratory syncytial virus	lower respiratory tract	M2, P, F, G ⁹
	Influenza virus	influenza	HA, M ¹⁰
	Adenovirus	common cold	
	rhinovirus	common cold	VP1, VP2, VP3 ¹¹
30	Parainfluenza virus	common cold	HN, F ¹²
	<i>Pneumocystis carinii</i>	pneumonia in AIDS	msg ¹³

- 1- (Flack et al., 1995 *Gene* 156:97-99; Panzutti et al., 1993, 61:1867-1872; Nelson et al., 1988, *Rev Infect Diseases* 10:S331-336).
- 2- (Pruksakorn et al., 1994, *Lancet* 344:639-642; Dole et al., 1993, *J Immunol* 151:2188-94).
- 5 3- (Murphy et al., 1989, *Infect Immun* 57:2938-2941; Faden et al., 1992, *Infect Immun* 60:3824-3829).
- 4- (Lock et al., 1992, *Microb Pathog* 12:137-143).
- 5- (Novotny et al., 1991, *Dev Biol Stand* 73:243-249;
- 10 Lipscombe et al., 1991, *Mol Microbiol* 5:1385-1392; He et al., 1993, *Eur J Clin Microbiol Infect Dis* 12:690-695).
- 6- (Rawling et al., 1995, *Infect Immun* 63:38-42; Pennington et al., 1988, *J Hosp Infect* 11A:96-102).
- 7- (Weeratna et al., 1994, *Infect Immun* 62:3454-3462).
- 15 8- (Jacobs et al., 1990, *Infect Immun* 58:2464-2469; 1990, *J Clin Microbiol* 28:1194-1197).
- 9- (Kulkarni et al., 1995, *J Virol* 69:1261-1264; Leonov et al., 1994, *J Gen Virol* 75:1353-1359; Garcia et al., 1993, *Virology* 195:239-242; Vaux-Peretz et al., 1992,
- 20 *Vaccine* 10:113-118).
- 10- (Kaly et al., 1994, *Vaccine* 12:753-760; Bucher et al., 1980, *J Virol* 36:586-590).
- 11- (Francis et al., 1987, *J Gen Virol* 68:2687-2691).
- 12- (Morein et al., 1983, *J Gen Virol* 64:1557-1569).
- 25 13- (Garbe et al., 1994, *Infect Immun* 62:3092-3101).

In another preferred embodiment, a microbial pathogen may include a pathogen causing a sexually transmitted disease selected from the group of

30 pathogens, with respective antigens, in Table 3.

Table 3

PATHOGEN	INFECTION/DISEASE	PROTEIN ANTIGEN
35 <i>N. gonorrhoeae</i>	gonorrhea	IgA1 protease ¹ , PIB ² , H.8 ³ , Por ⁴
<i>Chlamydia trachomatis</i>	nongonococcal urethritis	MOMP ⁵ , HSP ⁶

- 1- (Lomholt et al., 1994, *Infect Immun* 62:3178-83).
- 40 2- (Heckels et al., 1990, *Vaccine* 8:225-230).
- 3- (Blacker et al., 1985, *J Infect Dis* 151:650-657).
- 4- (Wetzler et al., 1992, *Vaccine* 8:225-230).
- 5- (Campos et al., 1995, *Ophthalmol Vis Sci* 36:1477-91; Murdin et al., 1995, *Infect Immun* 63:1116-21).
- 45 6- (Taylor et al., 1990, *Infect Immun* 58:3061-3).

Tables 2 & 3, and the references footnoted which are herein incorporated by reference, illustrate various protein antigens, or peptides thereof, viewed by those skilled in the art to be useful as vaccine candidates against the respective microbial pathogen. Typically, the immunopotency of an epitope, whether from a protein or peptide, of a microbial pathogen is determined by monitoring the immune response of an animal following immunization with the epitope and/or by analyzing human convalescent sera in conjunction with pre-immune sera. Thus, one skilled in the art can determine protein or peptide antigens from microbial pathogens which would be desired to include as a heterologous antigen to be expressed by an *htrB* mutant according to the present invention. A corresponding nucleic acid sequence, the encoding sequence, can then be deduced from the amino acid sequence of the protein or peptide antigen, wherein the encoding sequence is introduced into the *htrB* mutant for expression.

EXAMPLE 14

Use of *htrB* mutants to generate antisera

The *htrB* mutant, or endotoxin purified therefrom, can be used to generate endotoxin-specific antisera, directed to the particular gram-negative bacterial pathogen, which can be used in an immunoassay to detect the antigen (that particular gram-negative bacterial pathogen), present in the body fluid of an individual suspected of having an infection caused by that gram-negative bacterial pathogen. The body fluid(s) collected for analysis depend on the microorganism to be detected, the suspected site of infection, and whether the body fluid is suspected of containing the antigen or containing antisera. With those considerations in mind, the body fluid could include one or more of sputum, blood, cerebrospinal

fluid, lesion exudate, swabbed material from the suspected infection site, and fluids from the upper respiratory tract. Immunoassays for such detection comprises any immunoassay known in the art including, 5 but not limited to, radioimmunoassay, ELISA, "sandwich" assay, precipitin reaction, agglutination assay, fluorescent immunoassay, and chemiluminescence-based immunoassay.

Alternatively, where an immunocompromised 10 individual is suffering from a potentially life-threatening infection caused by a particular gram-negative bacterial pathogen, immunization may be passive, i.e. immunization comprising administration of purified human immunoglobulin containing antibody 15 against an *htrB* mutant or isolated *htrB* endotoxin of that particular gram-negative bacterial pathogen.

It should be understood that while the invention has been described in detail herein, the 20 examples were for illustrative purposes only. Other modifications of the embodiments of the present invention that are obvious to those skilled in the art of molecular biology, medical diagnostics, and related disciplines are intended to be within the scope of the 25 appended claims.

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- 5 (i) APPLICANT: Michael A. Apicella
Melvin G. Sunshine
Na-Gyong Lee
Bradley Gibson
Rasappa Arumugham
- 10 (ii) TITLE OF INVENTION: Non-Toxic Mutants of Pathogenic
Gram-Negative Bacteria
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
- 15 (A) ADDRESSEE: Hodgson, Russ, Andrews, Woods &
Goodyear
- (B) STREET: 1800 One M&T Plaza
- (C) CITY: Buffalo
- (D) STATE: New York
- 20 (E) COUNTRY: United States
- (F) ZIP: 14203-2391
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Diskette, 3.5 inch, 1.4 Mb
storage
- 25 (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: MS-DOS/ Microsoft Windows
3.1
- (D) SOFTWARE: Wordperfect for Windows 5.1
- (vi) APPLICATION DATA:
- 30 (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (vii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Nelson, M. Bud
- 35 (B) REGISTRATION NUMBER: 35,300
- (C) REFERENCE DOCKET NUMBER: 22244.0002
- (viii) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: (716) 856-4000
- (B) TELEFAX: (716) 849-0349
- 40 (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 969 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double-stranded
- 45 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: genomic DNA
- (iii) HYPOTHETICAL: yes
- (iv) ORIGINAL SOURCE:
- (A) ORGANISM: *H. influenzae*
- 50 (B) STRAIN: 2019
- (C) CELL TYPE: bacterium
- (v) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- TAAACTACGC CCCTAACTTA CGTGGAAAGA ACA ATG AAA AAC 42

Met Lys Asn

1

5	GAA	AAA	CTC	CCT	CAA	TTT	CAA	CCG	CAC	TTT	TTA	GCC	CCA	81
	Glu	Lys	Leu	Pro	Gln	Phe	Gln	Pro	His	Phe	Leu	Ala	Pro	
		5					10					15		
10	AAA	TAC	TGG	CTT	TTT	TGG	CTA	GGC	GTG	GCA	ATT	TGG	CGA	120
	Lys	Tyr	Trp	Leu	Phe	Trp	Leu	Gly	Val	Ala	Ile	Trp	Arg	
			20						25					
15	AGT	ATT	TTA	TGT	CTT	CCC	TAT	CCT	ATT	TTG	CGC	CAT	ATT	159
	Ser	Ile	Leu	Cys	Leu	Pro	Tyr	Pro	Ile	Leu	Arg	His	Ile	
	30					35					40			
20	GGT	CAT	GGT	TTC	GGT	TGG	CTG	TTT	TCA	CAT	TTA	AAA	GTG	198
	Gly	His	Gly	Phe	Gly	Trp	Leu	Phe	Ser	His	Leu	Lys	Val	
			45					50					55	
25	GGT	AAA	CGT	CGA	GCT	GCC	ATT	GCA	CGC	CGT	AAT	CTT	GAA	237
	Gly	Lys	Arg	Arg	Ala	Ala	Ile	Ala	Arg	Arg	Asn	Leu	Glu	
					60					65				
30	CTT	TGT	TTC	CCT	GAT	ATG	CCT	GAA	AAC	GAA	CGT	GAG	ACG	276
	Leu	Cys	Phe	Pro	Asp	Met	Pro	Glu	Asn	Glu	Arg	Glu	Thr	
		70					75					80		
35	ATT	TTG	CAA	GAA	AAT	CTT	CGT	TCA	GTA	GGC	ATG	GCA	ATT	315
	Ile	Leu	Gln	Glu	Asn	Leu	Arg	Ser	Val	Gly	Met	Ala	Ile	
			85						90				95	
40	ATC	GAA	ACT	GGC	ATG	GCT	TGG	TTT	TGG	TCG	GAT	TCA	CGT	354
	Ile	Glu	Thr	Gly	Met	Ala	Trp	Phe	Trp	Ser	Asp	Ser	Arg	
					100					105				
45	ATC	AAA	AAA	TGG	TCG	AAA	GTT	GAA	GGC	TTA	CAT	TAT	CTA	393
	Ile	Lys	Lys	Trp	Ser	Lys	Val	Glu	Gly	Leu	His	Tyr	Leu	
			110					115					120	
50	AAA	GAA	AAT	CAA	AAA	GAT	GGA	ATT	GTT	CTC	GTC	GGC	GTT	432
	Lys	Glu	Asn	Gln	Lys	Asp	Gly	Ile	Val	Leu	Val	Gly	Val	
					125					130				
55	CAT	TTC	TTA	ACG	CTA	GAA	CTT	GGC	GCA	CGC	ATC	ATT	GGT	471
	His	Phe	Leu	Thr	Leu	Glu	Leu	Gly	Ala	Arg	Ile	Ile	Gly	
		135					140					145		
60	TTA	CAT	CAT	CCT	GGC	ATT	GGT	GTT	TAT	CGT	CCA	AAT	GAT	510
	Leu	His	His	Pro	Gly	Ile	Gly	Val	Tyr	Arg	Pro	Asn	Asp	
				150					155					
65	AAT	CCT	TTG	CTT	GAT	TGG	CTA	CAA	ACA	CAA	GGC	CGT	TTA	549
	Asn	Pro	Leu	Pro	Asp	Trp	Leu	Gln	Thr	Gln	Gly	Arg	Leu	
	160					165					170			

CGC TCC AAT AAA GAT ATG CTT GAT CGT AAA GAT TTA CGC 588
Arg Ser Asn Lys Asp Met Leu Asp Arg Lys Asp Leu Arg 185

5 GGA ATG ATC AAA GCT TTA CGC CAC GAA GAA ACC ATT TGG 627
Gly Met Ile Lys Ala Leu Arg His Glu Glu Thr Ile Trp 190 195

10 TAT GCG CCT GAT CAC GAT TAC GGC AGA AAA AAT GCC GTT 666
Tyr Ala Pro Asp His Asp Tyr Gly Arg Lys Asn Ala Val 200 205 210

15 TTT GTT CCT TTT TTT GCA GTA CCT GAC ACT TGC ACT ACT 705
Phe Val Pro Phe Phe Ala Val Pro Asp Thr Cys Thr Thr 215 220

20 ACT GGT AGT TAT TAT TTA TTG AAA TCC TCG CAA AAC AGC 744
Thr Gly Ser Tyr Tyr Leu Leu Lys Ser Ser Gln Asn Ser 225 230 235

AAA GTG ATT CCA TTT GCG CCA TTA CGC AAT AAA GAT GGT 783
Lys Val Ile Pro Phe Ala Pro Leu Arg Asn Lys Asp Gly 240 245 250

25 TCA GGC TAT ACC GTG AGC ATT TCA GCG CCT GTT GAT TTT 822
Ser Gly Tyr Thr Val Ser Ile Ser Ala Pro Val Asp Phe 255 260

30 ACA GAT TTA CAA GAT GAA GTA GCG ATA GCT GTG CGA ATG 861
Thr Asp Leu Gln Asp Glu Val Ala Ile Ala Val Arg Met 265 270 275

35 AAT CAA ATC GTT GAA AAG GAA ATC ATG AAG GGC ATA TCA 900
Asn Gln Ile Val Glu Lys Glu Ile Met Lys Gly Ile Ser 280 285

40 CAA TAT ATG TGG CTA CAT CGT CGT TTT AAA ACA CGC CCC
Gln Tyr Met Trp Leu His Arg Arg Phe Lys Thr Arg Pro 290 295 300

GAT GAA AAT ACG CCT AGT TTA TAC GAT TAA 969
Asp Glu Asn Thr Pro Ser Leu Tyr Asp 305 310

45

- (3) INFORMATION FOR SEQ ID NO:2:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 31 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single-stranded
 - (D) TOPOLOGY: linear
- (ii) ORIGINAL SOURCE:
- (A) ORGANISM: *H. influenzae*
 - (B) STRAIN: 2019
- 50

- 63 -

- (iii) IMMEDIATE SOURCE: synthesized
(iv) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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5

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single-stranded
(D) TOPOLOGY: linear

10

(ii) ORIGINAL SOURCE:

- (A) ORGANISM: *H. influenzae*

(iii) FEATURE:

- (A) OTHER INFORMATION: uptake sequence for transformation

15

(iv) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAGTGCGGT 9

20

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single-stranded
(D) TOPOLOGY: linear

25

(ii) FEATURE:

- (A) OTHER INFORMATION: hybridizes to TNF α mRNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:4:

30

ATCTCTCAGC TCCACGCCAT TGGCCAGGAG 30

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single-stranded
(D) TOPOLOGY: linear

35

(ii) FEATURE:

- (A) OTHER INFORMATION: does not hybridize to TNF α mRNA

40

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCCTGGCCA ATGGCGTGGA GCTGAGAGAT 30

What is claimed is:

- 5 1. A method for immunizing an individual to prevent disease caused by a gram-negative bacterial pathogen, the method comprising vaccinating the individual with a prophylactically effective amount of a vaccine formulation comprising an active ingredient selected
10 from the group consisting of an *htrB* mutant of said gram-negative bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen said endotoxin
15 conjugated to a carrier protein, and an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous vaccine antigen; wherein said *htrB* mutant lacks one or more secondary acyl chains of lipid A
20 contained in the gram-negative bacterial pathogen resulting in substantially reduced toxicity when compared to lipid A of the gram-negative bacterial pathogen.
- 25 2. The method of claim 1, wherein the individual is a human, and the vaccine formulation is introduced by a route of administration selected from the group consisting of intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, ocular,
30 intranasal, and oral administration.
- 35 3. The method of claim 2, wherein the vaccine formulation comprises an active ingredient consisting essentially of an *htrB* mutant of said gram-negative bacterial pathogen.

4. The method of claim 2, wherein the vaccine formulation comprises an active ingredient consisting essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen.

5

5. The method of claim 2, wherein the vaccine formulation comprises an active ingredient consisting essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, wherein the isolated endotoxin is conjugated to a carrier protein.

10

6. The method of claim 2, wherein the vaccine formulation comprises an active ingredient consisting essentially of an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous antigen from a microbial pathogen.

15

7. The method of claim 2, wherein the vaccine formulation further comprises a physiological carrier and an adjuvant.

20

8. The method of claim 1, wherein the individual is an animal, and the vaccine formulation is introduced by a route of administration selected from the group consisting of intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, ocular, intranasal, and oral administration.

25

9. The method of claim 8, wherein the vaccine formulation comprises an active ingredient consisting essentially of an *htrB* mutant of said gram-negative bacterial pathogen.

30

10. The method of claim 8, wherein the vaccine formulation comprises an active ingredient consisting

35

essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen.

11. The method of claim 8, wherein the vaccine
5 formulation comprises an active ingredient consisting essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, wherein the isolated endotoxin is conjugated to a carrier protein.

10 12. The method of claim 8, wherein the vaccine formulation comprises an active ingredient consisting essentially of an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous antigen from a
15 microbial pathogen.

13. The method according to claim 8, wherein the *htrB* mutant of said gram-negative bacterial pathogen is administered orally as an additive to animal feed.

20 14. The method according to claim 12, wherein the *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous antigen from a microbial pathogen is
25 administered orally as an additive to animal feed.

15. The method of claim 8, wherein the vaccine formulation further comprises a physiological carrier and an adjuvant.

30 16. A vaccine formulation comprising an active ingredient selected from the group consisting of an *htrB* mutant of a gram-negative bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative
35 bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen said

endotoxin conjugated to a carrier protein, and an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous vaccine antigen; wherein said *htrB* mutant
5 lacks one or more secondary acyl chains of lipid A contained in the gram-negative bacterial pathogen resulting in substantially reduced toxicity when compared to lipid A of the gram-negative bacterial pathogen.

10

17. The vaccine formulation according to claim 16, wherein the active ingredient consists essentially of an *htrB* mutant of said gram-negative bacterial pathogen.

15

18. The vaccine formulation according to claim 16, wherein the active ingredient consists essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen.

20

19. The vaccine formulation of claim 16, wherein the active ingredient consists essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, wherein the isolated endotoxin is conjugated to a carrier protein.

25

20. The vaccine formulation according to claim 16, wherein the active ingredient consists essentially of an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at
30 least one heterologous antigen from a microbial pathogen.

35

21. The vaccine formulation according to claim 16, further comprising a physiological carrier and an adjuvant.

22. A method of making in a gram-negative bacterial pathogen a mutant endotoxin of substantially reduced toxicity when compared to the endotoxin of the wild type gram-negative bacterial pathogen, the method comprising
5 mutating an *htrB* gene within the gram-negative bacterial pathogen, wherein said mutation causes a phenotype of a resultant *htrB* mutant characterized by a mutant endotoxin lacking one or more secondary acyl chains of lipid A contained in the wild type gram-negative
10 bacterial pathogen.

23. A mutant endotoxin of substantially reduced toxicity, made according to the method of claim 22, wherein the mutant endotoxin having substantially
15 reduced toxicity was purified from the *htrB* mutant by a process selected from the group consisting of a phenol/water extraction, and a protease digestion; and wherein the purified mutant endotoxin having substantially reduced toxicity is used to generate
20 endotoxin-specific antibodies.

24. The mutant endotoxin according to claim 23, further comprising conjugation to a carrier protein.

25. A mutant endotoxin of substantially reduced toxicity, made according to the method of claim 22.

26. The mutant endotoxin according to claim 25, further comprising conjugation to a carrier protein.

27. A method of making an *htrB* mutant of a wild type gram-negative bacterial pathogen wherein the *htrB* mutant has substantially reduced toxicity when compared to the wild type gram-negative bacterial pathogen, the method
35 comprising mutating an *htrB* gene within the gram-negative bacterial pathogen, wherein said mutation

causes a phenotype of a resultant *htrB* mutant characterized by endotoxin lacking at least one secondary acyl chain on lipid A contained in the wild type gram-negative bacterial pathogen.

5

28. A mutant gram-negative bacterial pathogen of substantially reduced toxicity, made according to the method of claim 27, wherein the gram-negative bacterial pathogen having substantially reduced toxicity is used
10 to generate endotoxin-specific antibodies.

29. A method for producing endotoxin-specific antisera for a use selected from the group consisting of in diagnostic assays, and for passive immunization, the
15 method comprises immunizing an individual with a vaccine formulation comprising an active ingredient selected from the group consisting of an *htrB* mutant of said gram-negative bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative bacterial
20 pathogen, and endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen said endotoxin conjugated to a carrier protein; and collecting antibody produced from said immunized individual; wherein said *htrB* mutant lacks one or more secondary acyl chains of
25 lipid A contained in the wild type gram-negative bacterial pathogen resulting in substantially reduced toxicity when compared to lipid A of the wild type gram-negative bacterial pathogen.

30

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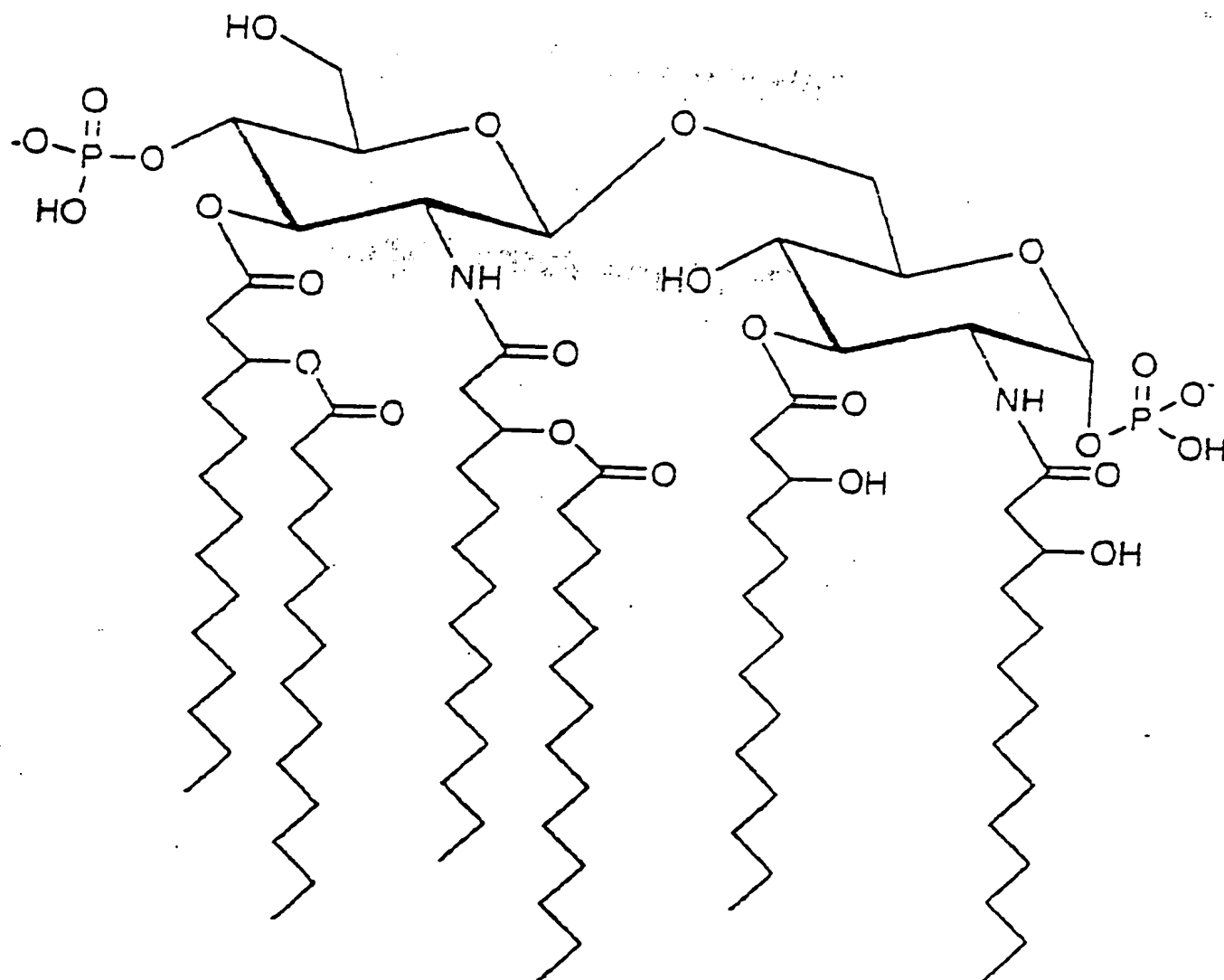


FIG. 1

3 Rec'd PCT/PTO 0 JUN 1998

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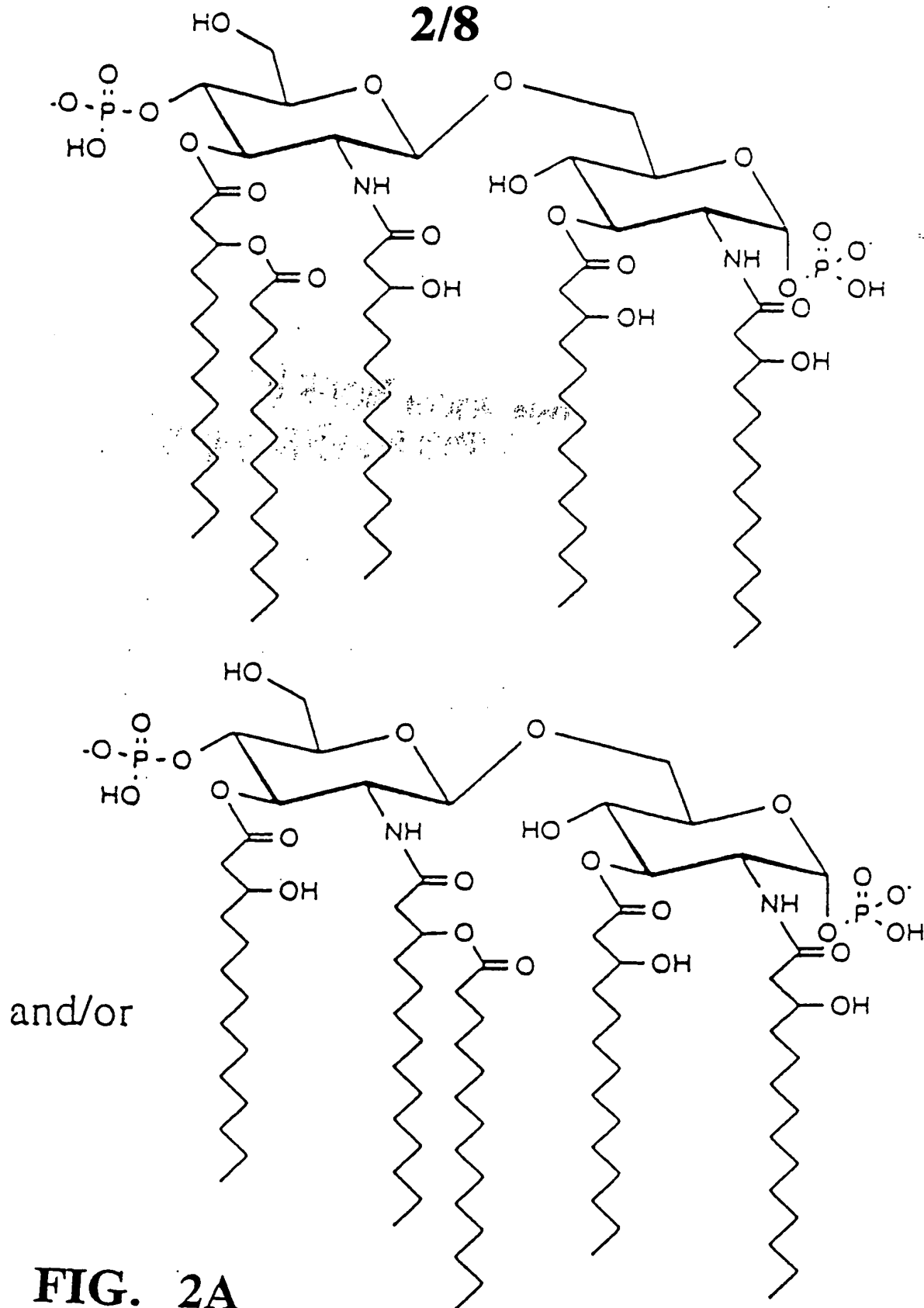


FIG. 2A

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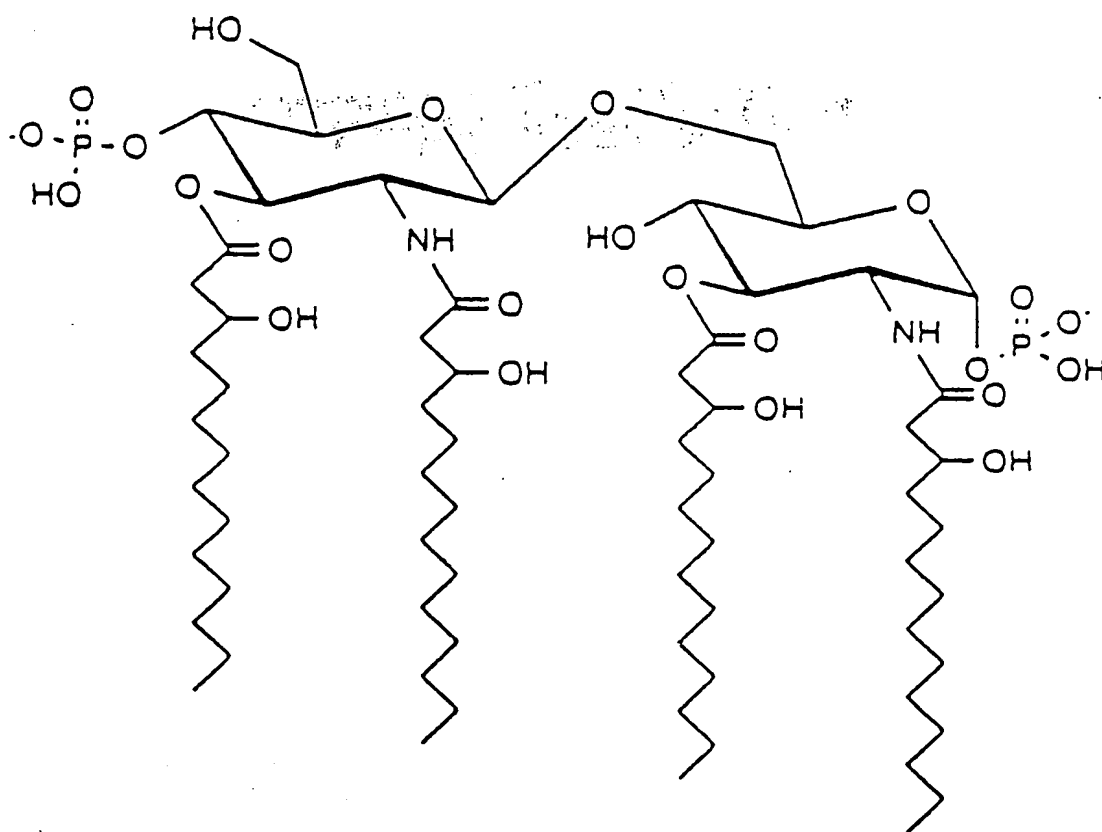


FIG. 2B

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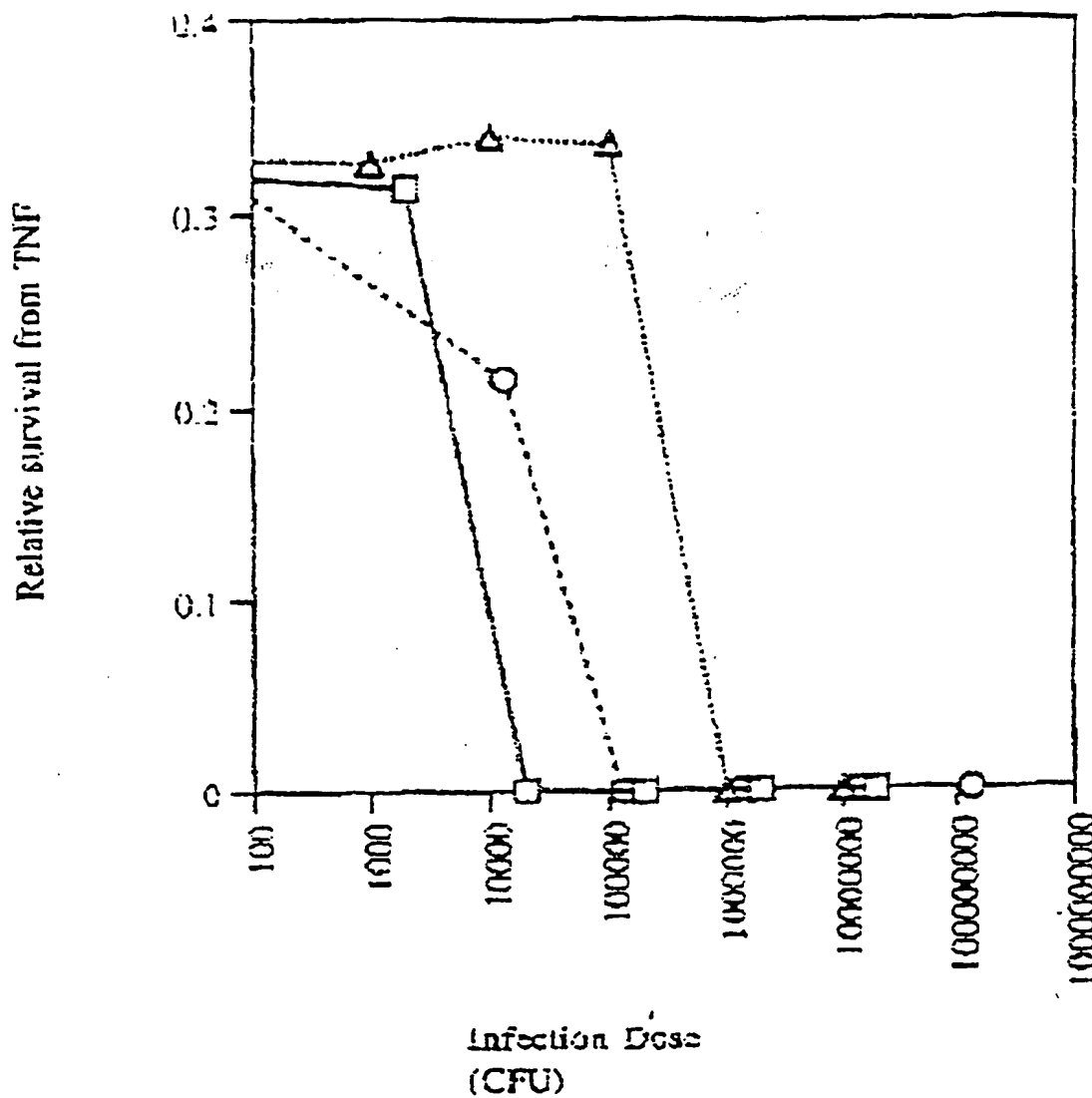


FIG. 3

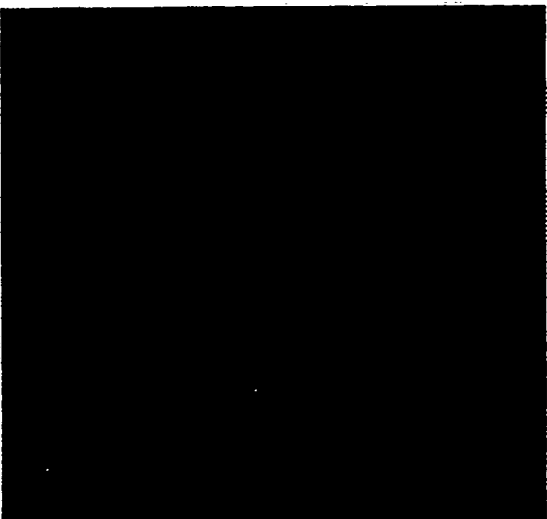
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5/8

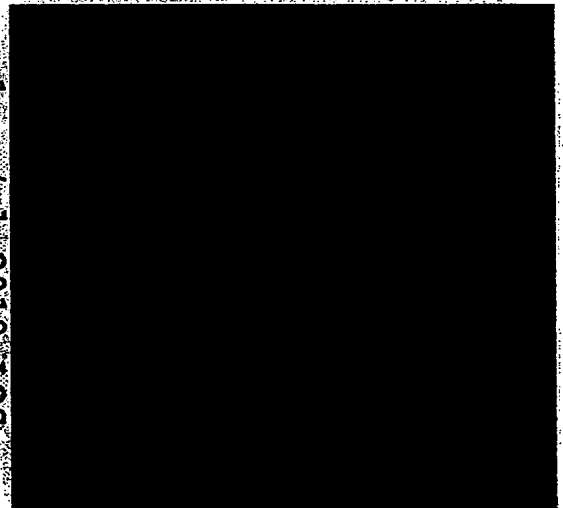
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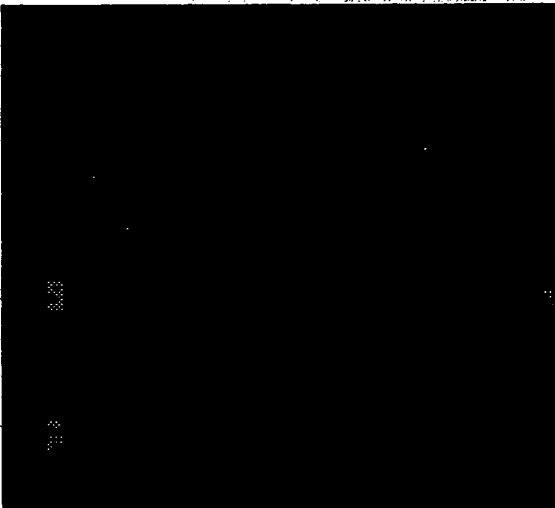
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No probe



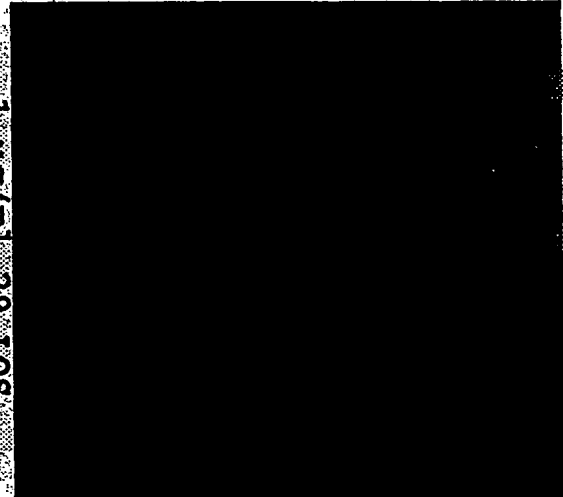
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Probe #1



1 ug/ml 2019-LOS
Probe #2



1 ug/ml B29-LOS
Probe #1



1 ug/ml B29-LOS
Probe #2

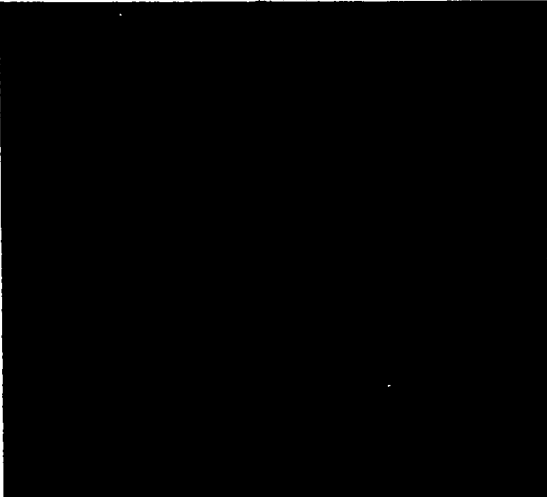


FIG. 4

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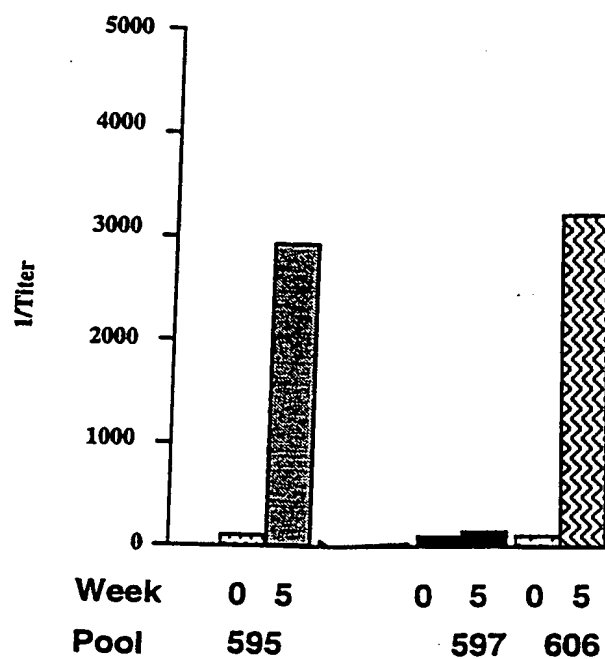


FIG. 5

[illegible]

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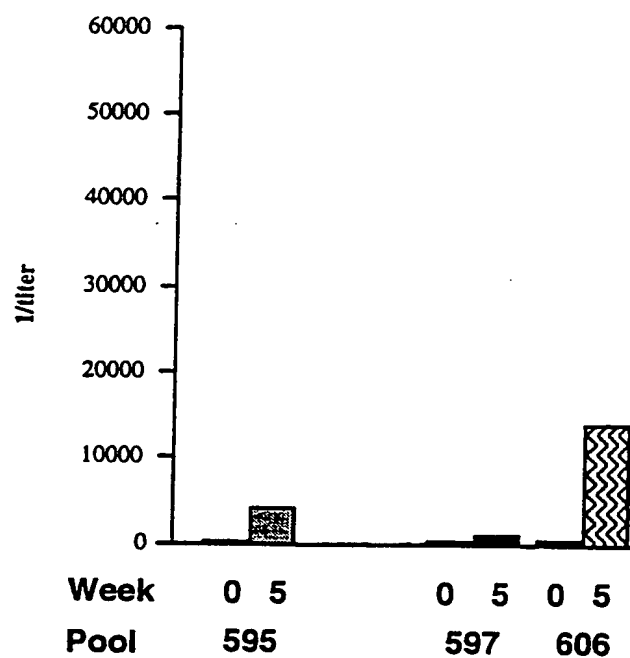


FIG. 6

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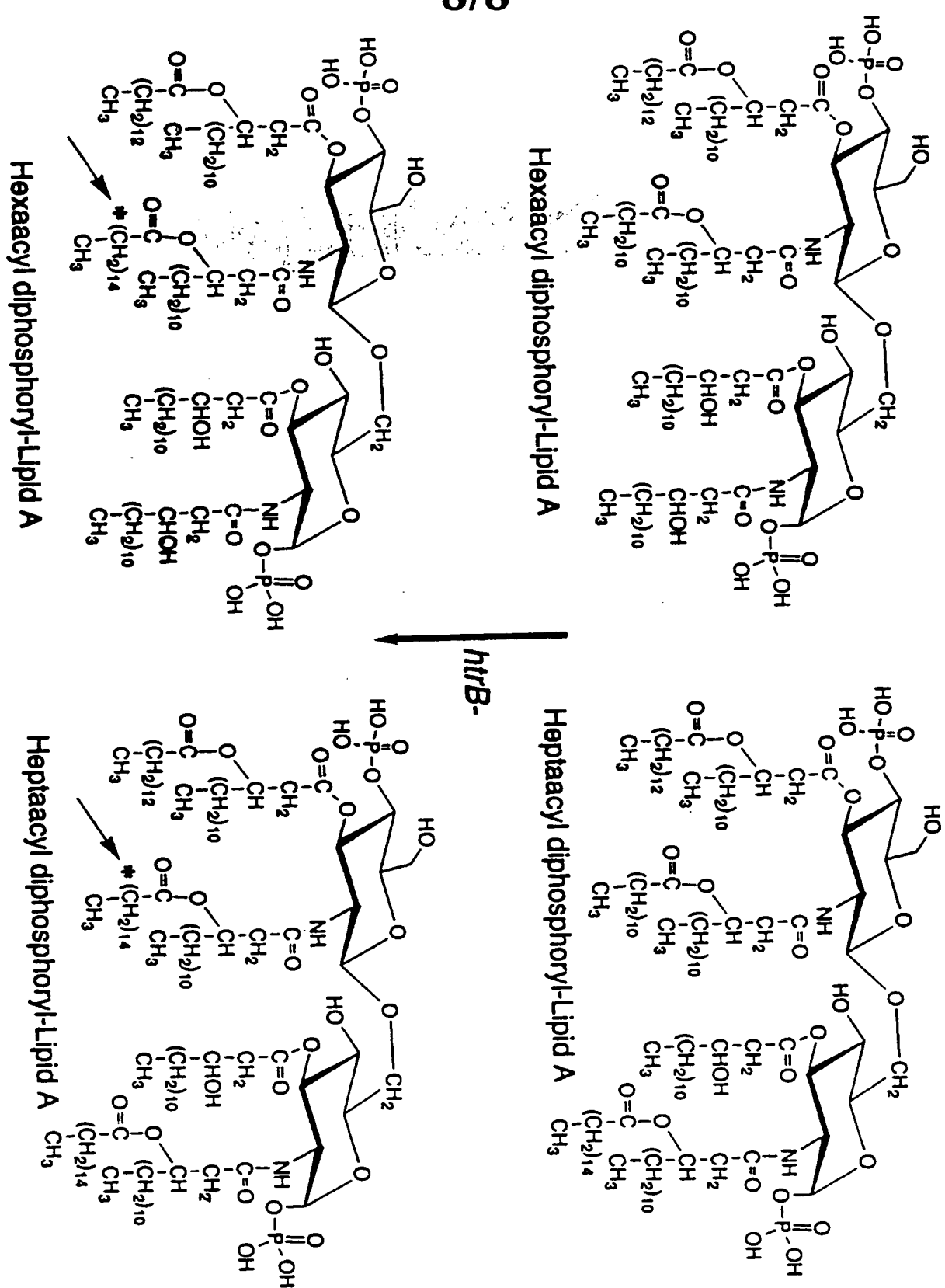


FIG. 7

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/18984

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 35/66, 39/00, 39/02; C07K 14/195

US CL : 424/9.2, 184.1, 234.1, 236.1; 530/403, 825

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.2, 184.1, 234.1, 236.1; 530/403, 825

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, MEDLINE, BIOSIS, HCAPLUS, WPIDS, EMBASE

search terms: endotoxin, gram-negative bacteria, htrb mutant, vaccine, pathogen

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,707,543 (ZOLLINGER et al.) 17 November 1987, see entire document.	1-16
Y	US, A, 4,912,094 (MYERS et al.) 27 March 1990, see entire document.	16-21
X	US, A, 4,929,604 (MUNFORD et al.) 29 May 1990, see entire document.	16-21
---		-----
Y		22-29
A	ZIEGLER et al. Treatment of Gram-Negative Bacteremia and Shock With Antiserum to a Mutant Escherichia Coli. 11 November 1982. Volume 307, Number 20, pages 1225-1230, see entire document.	1-29

☐

Further documents are listed in the continuation of Box C.

☐

See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A		document defining the general state of the art which is not considered to be of particular relevance
* B		earlier document published on or after the international filing date
* L		document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
* O		document referring to an oral disclosure, use, exhibition or other means
* P		document published prior to the international filing date but later than the priority date claimed
	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
	* &	document member of the same patent family

Date of the actual completion of the international search

30 JANUARY 1997

Date of mailing of the international search report

25 MAR 1997

Name and mailing address of the ISA/US
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Authorized officer

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Telephone No. (703) 308-0196

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(54) Title: NON-TOXIC MUTANTS OF PATHOGENIC GRAM-NEGATIVE BACTERIA (57) Abstract <p>A method is provided for identifying, isolating, and producing <i>htrB</i> mutants of gram-negative bacterial pathogens. The method comprises mutating the <i>htrB</i> gene of a gram-negative bacterial pathogen so that there is a lack of a functional <i>htrB</i> protein, resulting in a mutant that lacks one or more secondary acyl chains contained in the wild type gram-negative bacterial pathogen, and displays substantially reduced toxicity as compared to the wild type strain. Also, the present invention provides methods for using a vaccine formulation containing the <i>htrB</i> mutant, the endotoxin isolated therefrom, or the endotoxin isolated therefrom which is then conjugated to a carrier protein, to immunize an individual against infections caused by gram-negative bacterial pathogens by administering a prophylactically effective amount of the vaccine formulation.</p>		

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NON-TOXIC MUTANTS OF PATHOGENIC GRAM-NEGATIVE BACTERIA

This invention has been made with government support under grant AI 24616 awarded by the National Institutes of Health. The government has certain rights
5 in the invention.

Field of the Invention

The present invention relates to compositions comprising altered endotoxin (lipooligosaccharide (LOS);
10 lipopolysaccharide (LPS)) of gram-negative bacterial pathogens. More particularly, the present invention relates to the making of a form of endotoxin, by a genetically engineered gram-negative pathogen, which lacks a substantially toxic lipid A portion. Also
15 disclosed are prophylactic and therapeutic uses of the substantially detoxified endotoxin, and of mutant gram-negative bacteria producing the substantially detoxified endotoxin.

20 Background of the Invention

Gram-negative bacteria have an outer membrane comprised of components including proteins, lipoproteins, phospholipids, and glycolipids. The glycolipids comprise primarily endotoxin-lipopoly-
25 saccharides (LPS) or lipooligosaccharides (LOS), depending on the genus of bacteria. LPS are molecules comprised of

- a) a lipid A portion which consists of a glucosamine disaccharide that is substituted with phosphate groups
30 and long chain fatty acids in ester and amide linkages;
- b) a core polysaccharide which is attached to lipid A by an eight carbon sugar, KDO (ketodeoxyoctonoate), and heptose, glucose, galactose, and N-acetylglucosamine; and

c) an O-specific side chain comprised of repeating oligo-saccharide units which, depending on the genera and species of bacteria, may contain mannose, galactose, D-glucose, N-acetylgalactosamine, N-acetylglucosamine, L-rhamnose, and a dideoxyhexose (abequose, colitose, tyvelose, paratose, trehalose). LOS has a similar structure as LPS, containing a lipid A portion and a complex carbohydrate structure, but differs in that it does not contain repeating O-side chains.

The major antigenic determinants of gram-negative bacteria are believed to reside in the carbohydrate structure of the O-specific side chain of LPS and the complex carbohydrate structure of LOS. These carbohydrate structures may vary for different species of the same genera of gram-negative bacteria by varying one or more of the sugar composition; the sequence of oligosaccharides; the linkage between the oligosaccharides; and substitutions/modifications of an oligosaccharide (particularly a terminal oligosaccharide).

LPS and LOS have been considered as bacterial components which have potential as vaccine immunogens because of the antigenic determinants ("epitopes") residing in their carbohydrate structures. However, the chemical nature of LPS and LOS prevent the use of these molecules in vaccine formulations; i.e., active immunization with LPS or LOS is unacceptable due to the inherent toxicity of the lipid A portion. The pathophysiological effects induced (directly or indirectly) by lipid A of LPS or LOS in the bloodstream include fever; leucopenia; leucocytosis; the Schwartzman reaction; disseminated intravascular coagulation; abortion; and in larger doses, shock and death. Accordingly, there are no currently available vaccines which induce antibody responses to LPS or LOS epitopes.

As shown in FIG. 1, the lipid A portion of endotoxin generally comprises a hydrophilic backbone of glucosamine disaccharide which is either monophosphorylated or diphosphorylated (positions 1 and 4'); and which carries at least six molecules of ester- and amide-bound fatty acids. Four molecules of (R)-3-hydroxytetradecanoate (e.g. 3-hydroxy-myristoyl or β -hydroxymyristic acid or β -OH) are linked directly to the lipid A backbone at positions 2, 3, 2', and 3'. Hydroxyl groups of two of the four molecules of β -OH are substituted with normal fatty acids (termed "secondary acyl chains", and including dodecanoate, tetradecanoate, and hexadecanoate) in forming acyloxyacyl groups.

One approach to making a detoxified endotoxin molecule involves isolating the endotoxin, and enzymatically-treating the isolated endotoxin with a human neutrophilic acyloxyacyl hydrolase (U.S. Patent Nos. 4,929,604, 5,013,661 and 5,200,184). The acyloxyacyl hydrolase hydrolyzes the fatty acids (non-hydroxylated, secondary acyl chains) from their ester linkages to hydroxy groups of β -OH (hydroxylated). The resultant altered endotoxin, from enzymatic treatment, contained a lipid A moiety lacking non-hydroxylated fatty acids. This altered endotoxin exhibited reduced *in vivo* toxicity, but retained antigenicity.

Another approach involves a method of modifying isolated endotoxin by selectively removing the β -OH that is ester-linked to the reducing-end glucosamine backbone at position 3 (U.S. Patent No. 4,912,094; Reexamination B1 4,912,094). The selective removal of β -OH was accomplished using alkaline hydrolysis. The resultant modified endotoxin exhibited reduced *in vivo* toxicity, but retained antigenicity.

Both approaches involve chemically treating isolated endotoxin. Neither approach discloses the production in a gram negative bacterial pathogen of an

endotoxin having substantially reduced toxicity, yet retaining antigenicity. Further, there has been no disclosure of the use of a gram-negative bacteria, which has been engineered to produce an endotoxin having

5 substantially reduced toxicity and yet retaining antigenicity, in a prophylactic or therapeutic vaccine against endotoxic shock and gram-negative bacteremia.

Summary of the Invention

10 The present invention is directed to a method for producing, in a mutant gram-negative pathogen, LPS or LOS which exhibits substantially reduced toxicity as compared to the wild type endotoxin, and which retains the antigenicity of its corresponding wild type

15 endotoxin. The method comprises creating a mutation in the *htrB* gene of the gram-negative bacterial pathogen such that there is a lack of functional HtrB protein in the mutated gram-negative bacterial pathogen. It was found that lipid A produced by the *htrB* mutant lacks one

20 or both of the fatty acids (non-hydroxylated or secondary acyl chains) thereby rendering the endotoxin in an isolated form, or the mutant gram-negative bacterial pathogen bearing the endotoxin, substantially reduced in toxicity and yet retaining antigenicity, as

25 compared to wild type. Endotoxin isolated from *htrB* mutants, or the *htrB* mutants themselves (whole cell vaccine), can be used to immunize individuals at risk of gram-negative bacteremia by inducing antibodies to the major antigenic determinants which reside in the

30 carbohydrate structure of the O-specific side chain of LPS and the complex carbohydrate structure of LOS. Further, the *htrB* mutants can be engineered to express heterologous antigens of other microbial pathogens at the surface of the *htrB* mutant for presentation to a

35 vaccinated individual's immune system in a multivalent vaccine. Also, the endotoxin isolated from the *htrB*

mutants of the present invention may be used to generate LPS or LOS-specific antibody which may be useful for passive immunization and as reagents for diagnostic assays directed to detecting the presence of gram-negative bacterial pathogens in clinical specimens.

Brief Description of the Figures

- FIG. 1 is a schematic representation of the general structure of lipid A of gram negative bacteria of the family *Enterobacteriaceae*.
- FIG. 2A is a schematic representation of the general structure of a species of lipid A, from the LOS of an *htrB* mutant, comprising pentaacyl diphosphoryl lipid A.
- FIG. 2B is a schematic representation of the general structure of a species of lipid A, from the LOS of an *htrB* mutant, comprising tetraacyl diphosphoryl lipid A.
- FIG. 3 is a graph showing the relative toxicity of an *htrB* mutant (O, Δ) as compared to wild type bacteria (\square) in a $\text{TNF}\alpha$ release assay.
- FIG. 4 is a photograph showing human primary respiratory epithelial cells unstimulated (control), exposed to NTHi 2019 LOS, or exposed to *htrB* mutant B29 LOS, and reacted with either a fluorescent probe that hybridizes to $\text{TNF}\alpha$ mRNA (probe 1) or a fluorescent control probe (probe 2).
- FIG. 5 is a graph showing mean titers of anti-LOS antibody against NTHi 2019 LOS (antigen coating) in ELISA from mice immunized with NTHi 2019 (Pool 595), *htrB* mutant B29 LOS (Pool 597), or *htrB* mutant B29 LOS conjugated to a carrier protein (Pool 606), with adjuvant.
- FIG. 6 is a graph showing the mean titers of anti-LOS antibody against *htrB* mutant B29 LOS (antigen coating) in ELISA from mice immunized with NTHi 2019 (Pool 595), *htrB* mutant B29 LOS (Pool 597), or *htrB* mutant B29 LOS conjugated to a carrier protein (Pool 606), with adjuvant.

FIG. 7 is a schematic representation comparing the structures of wild type *Salmonella* lipid A and *htrB* mutant lipid A.

5

Detailed Description of the Invention

Definitions:

10 "Endotoxin" is a term used herein for purposes of the specification and claims to refer to the LPS or LOS of gram-negative bacterial pathogens, wherein the endotoxin is either in a cell-associated or isolated form. "htrB endotoxin" and "htrB mutant endotoxin" refer to endotoxin isolated and purified from an gram-negative
15 bacterial pathogen *htrB* mutant.

"vaccine candidate or vaccine antigen" is a term used herein for purposes of the specification and claims to refer to an endotoxin epitope having one or more of the following properties (a-d): (a) is immunogenic; (b)
20 is surface-exposed (which can be shown by techniques known in the art including immunofluorescence assays, electron microscopy staining procedures, and by bactericidal assays); (c) induces antibody having bactericidal activity in the presence of complement
25 and/or functions in immune clearance mechanisms; (d) induces antibody which neutralizes other functional activity of the epitope (immunogenicity, or toxicity, etc.).

"Gram-negative bacterial pathogen" is a term used
30 herein for the purposes of the specification and claims to refer to one or more pathogenic (to humans or animals) bacterium of a genus and species including *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Haemophilus ducreyi*, other
35 *Haemophilus* species, *Moraxella catarrhalis*, *Campylobacter jejuni*, *Salmonella typhimurium*, other

Salmonella species, *Shigella dysenteriae*, and other *Shigella* species, and *Pseudomonas aeruginosa*.

"Substantially reduced in toxicity" is a term used herein for the purposes of the specification and claims to refer to a reduction in bioactivity of at least 10 fold to 100 fold or more as compared to wild type endotoxin.

"Carrier protein" is a term used herein for the purposes of the specification and claims to refer to a protein which is conjugated to the *htrB* mutant endotoxin. While the *htrB* mutant endotoxin appears to be immunogenic on its own, it is known in the art that conjugation to a carrier protein can facilitate immunogenicity. Proteins which may be utilized according to the invention include any protein which is safe for administration to mammals and which may serve as an immunologically effective carrier protein. In particular embodiments, cell surface proteins, membrane proteins, toxins and toxoids may be used. Criteria for safety would include absence of primary toxicity and minimal risk of allergic reaction. Diphtheria and tetanus toxoids fulfill these criteria; that is, suitably prepared they are non-toxic, and the incidence of allergic reactions is acceptably low. Although the risk of allergic reaction may be significant for adults, it is minimal for infants.

According to additional particular embodiments of the invention, appropriate carrier proteins include, but are not limited to *Salmonella* flagellin, *Haemophilus* pilin, *Pseudomonas pili*, *Pseudomonas* exotoxin, outer membrane proteins of *Haemophilus* (15 kDa, 28-30 kDa, and 40 kDa membrane proteins) or *N. meningitidis* or *N. gonorrhoeae*, *Escherichia coli* heat labile enterotoxin LT_B, cholera toxin, pneumolysin of *S. pneumoniae*, and viral proteins including rotavirus VP7 and respiratory syncytial virus F and G proteins. Additionally, there

are many carrier proteins known in the art including, but not limited to, keyhole limpet hemocyanin, bovine serum albumin, and diphtheria toxin cross-reactive mutant protein ("CRM"). Additionally, there are several
5 methods known in the art for conjugating endotoxin to a carrier protein. Such methods may include, but are not limited to, the use of glutaraldehyde, or succinimidyl *m*-maleimidobenzoate, or 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide, or by using bromoacetylated
10 carrier protein (see, e.g. Robey et al., 1989, *Anal. Biochem.* 177:373-377). Conjugation of *htrB* endotoxin to a carrier protein toxin may reduce toxicity of the carrier protein toxin, but residual toxicity may remain. Further detoxification may be accomplished by means
15 known in the art such as employing formalin which reacts with free amino groups of the carrier protein toxin.

Alternatively, native carrier protein toxin may be detoxified with formalin to produce a conventional toxoid before conjugation to the *htrB* mutant endotoxin.
20 However, the prior formalin treatment reduces the number of free amino groups available for reaction during the conjugation process. CRM have an advantage in that they have no inherent toxicity yet none of their amino groups are occupied by the formalin. In the case of CRM197,
25 which is immunologically identical to native toxin, treatment with formalin (though there is no need to detoxify) greatly enhances the immunological response. It is thought that this is due to stabilization of the molecule against degradation by mechanisms of the body
30 and/or aggregation by cross-linking (immunogenicity of particles increases with size). While tetanus and diphtheria toxins are desirable carrier proteins, there may be other candidate carrier proteins which may also be suitable. Such other candidates for carriers include
35 toxins and proteins of pseudomonas, staphylococcus, streptococcus, pertussis, and *E. coli*.

Conjugation of endotoxin to carrier proteins can be performed by a variety of methods such as by direct conjugation to the carrier protein by cyanogen bromide, reductive amination, or by using bifunctional linkers. Such bifunctional linkers include, but are not limited to, N-hydroxy succinimide-based linkers cystamine, glutaraldehyde, and diamino hexane. *htrB* endotoxin is modified to contain sulfhydryl groups with the use of N-hydroxy succinimide-based linkers, or by the use of carbodiimide-mediated condensation of cystamine. The sulfhydryl-containing *htrB* endotoxin intermediates are then reacted to a carrier protein that has been derivatized with N hydroxy succinimidyl bromo acetate. Preferably, sulfhydryl groups are exposed on the conjugating *htrB* endotoxin for making a thiol linkage with the bromoacetylated carrier protein.

In a specific embodiment of the invention, *htrB* mutant endotoxin may be conjugated to a carrier protein, such as CRM, by using long chain sulfo N-succnimidyl 3-(2-pyridylthio)-propionate to thiolate the primary amino group(s) of the endotoxin. Long chain sulfo N-succnimidyl 3-(2-pyridylthio)-propionate was added to approximately 13 mg of the saccharide component of the *htrB* endotoxin in 0.1 M NaHCO₃ pH7.0 at a ratio of 1:1 (w/w) and incubated for an hour at room temperature. The mixture is then purified by gel filtration. The long chain sulfo N-succnimidyl 3-(2-pyridylthio)-propionate derivatized fractions were pooled. The N-pyridyl disulfides present in the derivatized fractions were reduced with 100 mM dithiothreitol, and purified by gel filtration. The thiolated endotoxin fractions were then collected. CRM197 was bromoacetylated by adding bromoacetic acid N hydroxy succinimide in a small volume of dimethyl formamide dropwise to CRM (in 0.1 M NaHCO₃) at a ratio of 1:1 (w/w) at 4°C. The solution was mixed and incubated for 1 hour at room temperature. The

reaction mixture was then purified by gel filtration, and the fractions containing bromoacetylated protein were collected. Derivatization of amino groups on carrier protein to bromoacetyl groups was monitored by a
5 decrease in the amount of free amino groups. Bromoacetyl CRM in 0.1 M NaHCO₃ was added to the thiolated *htrB* mutant endotoxin at a 1:1.5 ratio of protein to endotoxin (w/w) in 0.1 M NaHCO₃/1 mM EDTA, and the reaction was incubated overnight at 4°C. The final
10 conjugate was then be purified by gel filtration in a phosphate buffered saline pH 6.9.

The methods and compositions of the present invention relate to LPS and LOS biosynthetic pathways of
15 gram-negative bacterial pathogens. More specifically, the present invention relates to mutations in the *htrB* gene of gram-negative bacterial pathogens resulting in mutant bacteria bearing endotoxin which is substantially reduced in toxicity, and yet retains antigenicity, as
20 compared to wild type bacteria of the same species.

The genetics of lipid A biosynthesis of enteric bacteria, as it was known at the time of the present invention, is summarized in Schnaitman and Klena (1993, *Microbiol. Rev.* 57:655-682). Genes *lpxA*, *lpxB*,
25 *lpxC*, and *lpxD* encode gene products which function on the glucosamine backbone of lipid A including transfer of β -hydroxymyristic acid to glucosamine. The *htrB* gene was described as a gene that affects the inner core structure (KDO, heptose, phosphorylethanolamine (PEA))
30 which was discovered during a screen for genes necessary for growth of *Escherichia coli* at elevated temperatures. Knockout mutations of *htrB* resulted in mutant *E. coli* which exhibited a reduced sensitivity to deoxycholate, an inability to grow at temperatures above 32.5°C, and a
35 decrease in LPS staining intensity (Schnaitman et al., 1993, *supra*; Karow et al., 1992, *J. Bacteriol.* 174:7407-

7418). Karow et al. further noted that at between about 30°C to about 42°C, *E.coli htrB* mutants have changes in the fatty acid composition of both LPS and phospholipids, and particularly, overproduce
5 phospholipids, as compared to wild type. However, it was neither known nor suggested which one or more of the at least six molecules of ester- or amide bound fatty acids is lacking in the lipid A portion of LPS of *htrB* mutants. Also no mention was made that *htrB* mutants
10 contained a lipid A moiety specifically lacking one or both non-hydroxylated (secondary acyl chain) fatty acids responsible for endotoxicity; i.e. that the *htrB* mutant contained an altered endotoxin exhibiting reduced *in vivo* toxicity, but retaining antigenicity ("*htrB* endotoxin"), as compared to wild type.
15

The discoveries comprising the present invention include the unexpected results that knockout mutations of the *htrB* gene of gram-negative bacteria (including the family *Enterobacteriaceae*) result in *htrB*
20 mutants which specifically lack one or more secondary acyl chain fatty acids which are ester-bound to the hydroxyl groups of two of the four molecules of β -OH (as shown in FIG. 2). Thus, it appears that the HtrB protein has either acyltransferase activity, or
25 indirectly or directly affects regulation of acyltransferase activity. The following examples are presented to illustrate preferred embodiments of aspects of the present invention, and are not intended to limit the scope of the invention. In particular, a preferred
30 embodiment is the making of an *H. influenzae htrB* mutant, and methods of using the same as a whole cell, or to isolate therefrom the endotoxin, in vaccine preparations or to generate antibodies for therapeutic or diagnostic applications. However, since the lipid A
35 moiety is highly conserved among bacteria of the family *Enterobacteriaceae* and closely related gram-negative

bacteria, the invention relates to gram-negative bacterial pathogens, as defined previously herein. There is microheterogeneity in terms of the length of the secondary acyl chain (12 or 14 carbon chains) and to which of the four β -OH are substituted (1, 2, or 4) (Erwin et al., 1991, *Infect Immun* 59:1881-1887); however, the nature of the substitution is the same and thus the particular steps (genes and gene products) involved in the biosynthetic pathway appear conserved. For example, removal of secondary acyl chains from various gram-negative bacterial pathogens (*E. coli*, *H. influenzae*, *P. aeruginosa*, *S. typhimurium*, and *N. meningitidis*) using human acylxyacyl hydrolase resulted in deacylated LPS from all species tested having significantly reduced mitogenic activity (Erwin et al., 1991, *supra*) as compared to the respective wild type strain.

EXAMPLE 1

Identification of an *htrB* gene, and generation of *htrB* mutants

By complementing a nontypable *H. influenzae* strain 2019 with a *S. typhimurium rfaE* mutant strain, the *rfaE* gene of *H. influenzae* strain 2019 was cloned (Lee et al., 1995, *Infect Immun* 63:818-824). Sequence analysis of the upstream region of the *H. influenzae rfaE* gene revealed an open reading frame highly homologous to the *E. coli htrB* gene. The *H. influenzae htrB* gene comprises 933 bases and encodes a protein, HtrB, of 311 amino acids (SEQ ID NO:1) and an estimated molecular size of 36 kilodaltons (kDa). Comparison of the deduced amino acid sequence of the *H. influenzae* HtrB with the *E. coli* HtrB revealed shared homology (56% identity and 73% similarity). Cloning the *htrB* gene of *H. influenzae* into a plasmid, and subsequent *in vitro*

transcription-translation analysis, revealed that HtrB has an apparent molecular size of 32-33 kDa.

There are various standard techniques known to those skilled in the art for mutating a bacterial gene. Those techniques include site-directed mutagenesis, and shuttle mutagenesis using transposons. In one aspect of this embodiment, mutagenesis of the *htrB* gene was carried out by shuttle mutagenesis. A derivative of the bacterial transposon Tn3, mini-Tn3 (Seifert et al., 1986, *Proc. Natl. Acad. Sci. USA* 83:735-739), was used as an insertion sequence to mutate the *htrB* gene. A 2.4 kilobase (kb) *Bgl*III containing the *htrB* gene from *H. influenzae* was cloned into a plasmid which was used as a target for mini-Tn3 transposon mutagenesis. Briefly, introduced into a single bacterial cell (*E. coli*), is the plasmid containing the *htrB* gene; a plasmid immune to Tn3 transposition and containing transposase (which mediates the cointegration between Tn3 and the target molecules); and a plasmid containing mini-Tn3.

After allowing for transposition, the bacterial cells are mated with an *E. coli* strain containing the *cre* enzyme that is used to resolve cointegrates in shuttle mutagenesis. Transconjugates were selected for with antibiotics (kanamycin, ampicillin, and streptomycin) and analyzed by restriction endonuclease digestion.

Two plasmids, termed pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location. Each plasmid was used to transform nontypable *H. influenzae* strain 2019 and bacterial cell transformants were selected for by growth in the presence of chloramphenicol (1.5 µg/ml), resulting in identification of mutant strains designated NTHi B28 and B29, respectively. Locations of the mTn3 insertion in the chromosomes of the NTHi mutants were

confirmed by genomic Southern hybridization using the 2.4 kb *Bgl*III fragment as a probe. In particular, a *Bgl*III digest of NTHi strain 2019 DNA resulted in a 2.4 kb fragment; whereas similar digests of DNA from mutants
5 NTHi B28 and B29 revealed 4.0 kb fragments. Further, the 4.0 kb fragments were digested by *Eco*RI which is present in the mTn3.

Alternatively, methods are known in the art to perform site-directed mutagenesis into a bacterial gene
10 (See for example, Halladay, 1993, *J. Bacteriol.* 175:684-692), and recombination of the mutated bacterial gene into the bacterial chromosome. A selectable kanamycin resistance cassette may be used to insert into, and mutate, the *htrB* gene contained within a shuttle
15 plasmid. Subsequent transformation into a bacterial host cell with the shuttle plasmid, and recombination of the bacterial genome (at the site of the genomic copy of the *htrB* gene) with the cassette via *htrB* flanking sequences, results in the site-directed mutagenesis of
20 the bacterial *htrB* gene.

Primer extension analysis can be used to determine the promoter region of the *htrB* gene. The *H. influenzae htrB*'s promoter region was determined by primer extension analysis by first growing the bacteria,
25 harvesting and purifying the RNA, and using a commercial primer extension kit according to the manufacturer's suggestions. A single transcription site was found using a primer (SEQ ID NO:2) complementary to the 5' region of the *htrB* open reading frame. The first
30 nucleotide was a cytosine (C) residue located at 21 bp upstream of the putative translation start site, ATG. The region upstream of the transcription start site contained a sequence (SEQ ID NO:1, bases 13 to 29) similar to the consensus -10 region of the bacterial σ^{70} -
35 dependent promoters at an appropriate distance. An

element (SEQ ID NO:1, bases 1 to 6) resembles the consensus sequence of the -35 region.

EXAMPLE 2

5 Characterization of *htrB* mutants

Growth Characteristics

NTHi B28 and B29 strains were initially selected at 30°C, and were unable to grow at 37°C. With further passages at 30°C, the NTHi *htrB* mutants began to
10 lose temperature sensitivity and demonstrated a slow rate of growth, as compared to NTHi 2019, at 37°C. However, for growth temperatures greater than or equal to 38.5°C, the temperature sensitivity remained for the *htrB* mutants.

15 It was reported previously that *E. coli htrB* mutants demonstrated a change in membrane permeability to various compounds including kanamycin and deoxycholate (Karow et al., 1992, *supra*). The NTHi *htrB* mutants were also tested for sensitivity to kanamycin
20 and deoxycholate. Overnight cultures grown at 30°C were then diluted and allowed to grow in the presence of 5 µg/ml kanamycin at either 30°C or 37°C. At 30°C, no difference was detected in the growth rate between NTHi 2019 and the NTHi *htrB* mutant strains in the absence of
25 kanamycin. However, the growth of the *htrB* mutants was significantly inhibited in the presence of kanamycin, whereas NTHi 2019 was not affected. For the *htrB* mutants, the sensitivity to kanamycin was even greater at 37°C, since the mutants failed to show growth in the
30 presence of kanamycin at 37°C. Likewise, at 30°C the *htrB* mutants showed sensitivity, as compared to NTHi strain 2019, at concentrations of greater than 500 µg/ml deoxycholate, and failed to grow at 1000 µg/ml. At
35 37°C, the *htrB* mutants showed almost complete inhibition of growth in the presence of only 250 µg/ml deoxycholate.

Endotoxin Characteristics

The LPS of *E. coli htrB* mutants has been characterized as being weakly stained on silver-stained polyacrylamide gels, but its migration pattern did not vary as compared to LPS from wild type. In contrast, the LOS from NTHi mutants B28 and B29 migrated faster than that from NTHi strain 2019 on silver-stained gels. Additionally, the LOS from the B28 and B29 mutants displayed a brownish color rather than black, as displayed by NTHi 2019. Reconstitution, by introducing a plasmid with an intact *htrB* gene into the mutant, of NTHi mutant B29 confirmed that the differences in growth characteristics and LOS migration and staining were due to mutation of the *htrB* gene.

The NTHi *htrB* mutant LOS and wild type LOS were each analyzed by electrospray ionization-mass spectrometry (ESI-MS) to provide molecular mass profiles for the different components of LOS. First, LOS was isolated from the respective strains. LPS or LOS can be isolated by the phenol-water method (Westphal et al., 1965, *Methods in Carbohydrate Chemistry* 5:83-91); or using an alternative purification procedure (using a protease; Hitchcock et al., 1983, *J. Bacteriol.* 154:269-277). The isolated LOS species were then O-deacylated by mild hydrazine treatment (37°C for 20 minutes; see Phillips et al., 1990, *Biomed. Environ. Mass Spectrom.* 19:731-745). Analysis by ESI-MS of the different LOS species showed that while the O-deacylated LOS from NtHi mutant B29 and NTHi 2019 were similar in molecular mass profile, two differences can be clearly discerned. In the *htrB* mutant, there is a decrease (50% reduction) in the amount of LOS containing two phosphoethanolamines (PEA) in the inner core structure; and there is a shift to high molecular weight LOS species containing more hexoses. These findings suggest that the degree of phosphorylation may be affecting chain progression from

specific heptose moieties, and that HtrB either directly or indirectly affects phosphorylation of LOS.

Mass spectrometry was used to analyze the lipid A. More specifically, lipid A from *htrB* mutant
5 LOS and from wild type LOS were each analyzed by liquid secondary ion mass spectrometry (LSIMS) in the negative ion mode to provide a spectrum of molecular ions for the different components lipid A. First, the LOS species were each hydrolyzed in 1% acetic acid for 2 hours at
10 100°C at a concentration of 2 mg/ml. The hydrolysates were centrifuged, and the supernatants removed. The water soluble crude lipid A fractions were washed twice in water, and once in an organic mixture (chloroform/methanol/water; by volume 2:1:1) and then
15 evaporated to dryness. For analysis, the lipid samples were redissolved in CH₂Cl₂/CH₃OH (3:1, v/v) and 1 µl of nitro-benzylalcohol/triethanolamine (1:1, v/v) and applied as a liquid matrix onto a mass spectrometer. LSIMS of the wild type (NTHi 2019) revealed a spectrum
20 containing two deprotonated molecular ions consistent with a hexaacyl lipid A structure containing either one (hexaacyl monophosphoryl lipid A, M_r = 1744) or two phosphates (hexaacyl diphosphoryl lipid A, M_r = 1824). This spectrum is essentially identical to that reported
25 for the lipid A structure of LOS of *H. ducreyi* (Melaugh et al., 1992, *J. Biol. Chem.* 267:13434-13439). The lower mass fragments are believed to be ions which arise through LSIMS-induced fragmentation of higher mass mono- and diphosphorylated molecular ion species.

30 In contrast, the LSIMS spectrum for the lipid A preparation from the *htrB* mutant LOS lacks molecular ions corresponding to the wild type hexaacyl lipid A species. There are two high mass ions which correspond to the molecular ions for a mono- and diphosphoryl
35 pentaacyl lipid A species missing one of the secondary acyl chains (e.g. myristic acid moiety). Further, at

the lower masses are two additional molecular ion species that correspond to a mono- and diphosphoryl tetraacyl lipid A species lacking both secondary acyl chains. In summary, the lipid A structure of the wild type's LOS is hexaacyl; whereas the lipid A structure of the *htrB* mutant shows two species, a tetraacyl (Fig. 2A) and a pentaacyl species (FIG. 2B) indicating the loss of at least one, and sometimes both secondary acyl chains. The *htrB* mutant is comprised of approximately 90% tetraacyl lipid A with only the four hydroxymyristic acid ester and amide-linked fatty acids, and approximately 10% pentaacyl lipid A with one myristic acid substitution.

15

EXAMPLE 3

Substantially reduced toxicity of *htrB* mutants

The effect due to the lack of one or more secondary acyl chains on the toxicity of a gram-negative bacterial pathogen was examined using a standard *in vitro* assay for measuring *in vivo* toxicity. Murine macrophage-like cell line J774, when stimulated by endotoxin, secretes TNF α . The amount of TNF α , a directly proportional to the toxicity of the stimulating LPS or LOS, can be measured by (a) removing the cell-free supernatant containing the TNF α ; (b) adding the supernatant to a TNF α -sensitive cell line, such as WEHI 164; and (c) measuring the resultant cytotoxicity (See for example, Espevik et al., 1986, *J Immunol Methods* 95:99; Sakurai et al., 1985, *Cancer Immunol Immunother* 20:6-10; Adams et al., 1990, *J Clin Microbiol* 28:998-1001; Adams et al., 1990, *J Leukoc Biol* 48:549-56; Tsai et al., 1992, *Cell Immunol* 144:203-16; and Pfister et al., 1992, *Immunol* 77:473-6).

In this assay, adherent J774 cells were removed from culture, washed with PBS-1mM EDTA, and then washed twice with complete tissue culture medium without

antibiotics. 2×10^6 to 4×10^6 J774 cells/100 mm culture dish were incubated in tissue culture medium overnight in a CO₂ incubator. Adherent J774 cells are removed with PBS-1mM EDTA, washed three times in tissue culture medium, and adjusted to 5×10^5 /ml. Aliquots of 50 μ l were added per well of a round bottom 96 well plate. The plate is then incubated for 1 hour at 37°C in a CO₂ incubator. Per well is added either an *htrB* mutant, or the wild type strain, in various colony forming units (cfu, infection dose). The plate is then incubated at 37°C for 1 hour in a CO₂ incubator. After the incubation, 100 μ l of culture medium containing 50 μ g/ml gentamycin is added per well. The plate is then incubated overnight at 37°C in a CO₂ incubator. Aliquots of 50 μ l of the J774 supernatant were removed per well and transferred into wells of a flat bottom 96 well plate. Serial 10 fold dilutions were made of the J774 supernatant. Included as a control is a dilution series of recombinant TNF α (rTNF α). Added per well is 50 μ l of WEHI 164 clone 13 cells at 6×10^5 cells/ml in tissue culture medium + 25 mM LiCl + 2 μ g/ml actinomycin D; and the mixture was incubated overnight at 37°C in a CO₂ incubator. After the incubation, 10 μ l of alomar blue is added, and 5-7 hours later the optical density is read at 570 nm. The assay utilizes alomar blue as a color indicator; i.e., alomar blue is converted to a red color by living cells, but remains blue if the cells are killed.

FIG. 3 shows a comparison between the number of bacterial cells of *H. influenzae* strain 2019 (wild type, \square), and of bacterial cells of *htrB* mutant NTHi B29 (O and Δ) necessary to stimulate the release of enough TNF α from J774 cells to kill the TNF α -susceptible cell line WEHI 164. B29_{hi} (Δ) and B29_{lo} (O) refer to a high number (>3) and low number (<3) of passages of *htrB*

mutant, respectively. As shown in FIG. 3, the *htrB* mutant shows a reduced ability to stimulate TNF α release; i.e., between an approximately 10 fold reduction (B29_{Lo}) to an approximately 100 fold reduction (B29_M). This reduced ability to stimulate TNF α is one indication of the *htrB* mutant being substantially reduced in toxicity due to the lack of one or more secondary acyl chains in the lipid A portion of the endotoxin.

The effect due to the lack of one or more secondary acyl chains on the toxicity of a gram-negative bacterial pathogen was also examined using a standard *in situ* assay for measuring *in vivo* toxicity. SV-40 transformed human respiratory epithelial cells and human primary respiratory epithelial cells, when stimulated by endotoxin, produces TNF α which production can be demonstrated by detection of TNF α mRNA using methods known to those skilled in the art for *in situ* hybridization (a modification of MacNaull et al., 1990, *J. Immunol.* 145:4154-66). The cells are grown in a monolayer within wells of a 24-well plate until approximately confluent. To stimulate the cells, 1 μ g/ml of LOS is added, and the cells are incubated overnight at 37°C. A counterstain (e.g., membrane stain) is added and the cells are then fixed with 0.5 ml of 2% paraformaldehyde in buffer so that the counterstain is fixed directly into the cells. Hybridization is then performed using an oligonucleotide probe specific for TNF α RNA (SEQ ID NO:4), and a control probe (SEQ ID NO:5). The amount of TNF α mRNA visually detected is directly proportional to the toxicity of the stimulating LPS. TNF α mRNA was minimally induced in SV-40 transformed human respiratory epithelial cells, and human primary respiratory epithelial cells (FIG. 4), exposed to LOS isolated from the *htrB* mutant NTHi B29. This is in contrast to LOS isolated from parent strain NTHi 2019 which induced high

levels of TNF α mRNA in these human respiratory cells (FIG. 4) and cell lines.

The substantial reduction in toxicity exhibited by the *htrB* mutant, as observed by the TNF α assays, due to the lack of one or more secondary acyl chains is further supported by previously reported assays of bioactivity of endotoxin treated with acyloxyacyl hydrolase which selectively removes the secondary acyl chains from endotoxin. Deacylated endotoxin from *E. coli*, *H. influenzae*, *N. meningitidis*, and *S. typhimurium* were (a) similarly reduced in potency in the Limulus lysate test relative to the respective wild type endotoxin; (b) reduced in the ability to stimulate neutrophil adherence to human endothelial cells relative to the respective wild type endotoxin; and (c) reduced in mitogenic activity for murine splenocytes (Erwin et al., 1991, *Infect Immun* 59:1881-1887); yet maintained expression of antigenic epitopes. Similarly, *S. typhimurium* LPS treated with acyloxacyl hydrolase showed a reduction in toxicity by 100-fold or greater in a dermal Shwartzman reaction; was less pyrogenic in a thermal response model; showed a 5 to 12 fold reduction in B-cell mitogenicity; and showed a 10 to 20 fold reduction in the release of prostaglandin E₂, as compared to wild type endotoxin, in concluding that maximally deacylated LPS was at least 10 fold less toxic than wild type endotoxin (U.S. Patent No. 4,929,604).

EXAMPLE 4

Use of *htrB* mutants as immunogens

In one aspect of this embodiment, the *htrB* mutant of a gram-negative bacterial pathogen is used as a whole cell vaccine. The benefit of using live, attenuated (weakened in its ability to cause pathogenesis) bacteria as an immunogen in a vaccine formula is that they are able to survive and may persist.

in the human or animal body, and thus confer prolonged immunity against disease. In conjunction with the benefit of using a live bacteria to prolong the immune response, gram-negative bacterial pathogen *htrB* mutants have the added benefit in that they exhibit substantially reduced toxicity. Another advantage, as compared to a vaccine formulation comprising an isolated peptide representing a bacterial antigen, is that a bacterial antigen expressed on the surface of a bacterial cell will often result in greater stimulation of the immune response. This is because the surface of bacteria of the family *Enterobacteriaceae* acts as a natural adjuvant to enhance the immune response to an antigen presented thereon (Wezler, 1994, *Ann NY Acad Sci* 730:367-370). Thus, using a live bacterial vaccine, such as an *htrB* mutant, to express complete proteins in an native conformation (i.e., as part of the bacterial outer membrane) is likely to elicit more of a protective immune response than an isolated protein alone.

Live bacterial vaccine vectors of the family *Enterobacteriaceae* that have been described previously include attenuated *Salmonella* strains (Stocker et al., U.S. Patent Nos. 5,210,035; 4,837,151; and 4,735,801; and Curtiss et al., 1988, *Vaccine* 6:155-160; herein incorporated by reference), and *Shigella flexneri* (Sizemore et al., 1995, *Science* 270:299-302; herein incorporated by reference). One preferred embodiment is to provide a vaccine delivery system for human or animal (depending on the genus and species of the gram-negative bacterial pathogen) mucosal pathogens. Thus, immunization by the parental route or by the mucosal route with a prophylactically effective amount of the *htrB* mutant, or an *htrB* mutant transformed to recombinantly express additional bacterial antigens (that do not negatively affect the growth or replication of the transformed *htrB* mutant), can lead to

colonization of mucosal surfaces to induce mucosal immunity against the antigens displayed on the surface of, or secreted from the *htrB* mutant. The resultant *htrB* mutant can be used in a vaccine formulation which
5 expresses the bacterial antigen(s).

Similar methods can be used to construct an inactivated *htrB* mutant vaccine formulation except that the *htrB* mutant is inactivated, such as by chemical means known in the art, prior to use as an immunogen and
10 without substantially affecting the immunogenicity of the expressed immunogen(s). For example, human bronchial mucosal immunity has been stimulated with an aerosol vaccine comprising lysed *H. influenzae* (Latil et al., 1986, *J Clin Microbiol* 23:1015-1021). Either of
15 the live *htrB* mutant vaccine or the inactivated *htrB* mutant vaccine may also be formulated with a suitable adjuvant in order to further enhance the immunological response to the antigen(s) expressed by the vaccine vector, as to be described in more detail.

20 In another aspect of this embodiment, the endotoxin is isolated from the *htrB* mutant using methods known in the art, and the isolated *htrB* endotoxin is used in a vaccine formulation. As mentioned previously, major antigenic determinants of gram-negative bacteria
25 are believed to reside in the carbohydrate structure of the O-specific side chain of LPS and the complex carbohydrate structure of LOS. However, the chemical nature of LPS and LOS prevent the use of these molecules in vaccine formulations; i.e., active immunization with
30 LPS or LOS is unacceptable due to the inherent toxicity of the secondary acyl chains of the lipid A portion of endotoxin. The endotoxin isolated from an *htrB* mutant of a gram-negative bacterial pathogen lacks one or more secondary acyl chains, and thus exhibits substantially
35 reduced toxicity as compared to endotoxin isolated from the respective wild type bacteria. Therefore, endotoxin-

isolated from an *htrB* mutant of a gram-negative bacterial pathogen can be used in a vaccine formulation in inducing immunity against the respective wild type gram-negative bacterial pathogen. LPS or LOS can be
5 isolated by the phenol-water method (Westphal et al., 1965, *Methods in Carbohydrate Chemistry* 5:83-91); or using an alternative purification procedure (using a protease; Hitchcock et al., 1983, *J. Bacteriol.* 154:269-277).

10 Many methods are known for the introduction of a vaccine formulation into the human or animal (collectively referred to as "individual") to be vaccinated. These include, but are not limited to, intradermal, intramuscular, intraperitoneal,
15 intravenous, subcutaneous, ocular, intranasal, and oral administration. Conventionally, vaccine formulations containing either live bacteria, or attenuated or inactivated bacteria, are administered by injection or by oral administration. For example, respiratory
20 immunity can be stimulated by intestinal immunization with purified *H. influenzae* antigens (Cripps et al., 1992, *J. Infect Dis* 165S1:S199-201; herein incorporated by reference). The vaccine formulation may comprise a physiologically acceptable solution as a carrier in
25 which the *htrB* mutant bacterial cells or isolated *htrB* mutant endotoxin is suspended. Various adjuvants may be used in conjunction with vaccine formulations. The adjuvants aid by modulating the immune response and in attaining a more durable and higher level of immunity
30 using smaller amounts of vaccine antigen or fewer doses than if the vaccine antigen were administered alone. Examples of adjuvants include incomplete Freund's adjuvant, Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate), oil emulsions,
35 Ribi adjuvant, the pluronic polyols, polyamines, Avridine, Quil A, saponin, MPL, QS-21, and mineral gels.

such as aluminum hydroxide, aluminum phosphate, etc. The vaccine formulation is administered in a prophylactically effective amount to be immunogenic, which depends on factors including the individual's
5 ability to mount an immune response, the degree of protection to be induced, and the route of administration.

In another aspect of the invention, the vaccine formulation can be administered orally by
10 including it as part of the feed given to economically important livestock. As known by those skilled in the art, species of *Haemophilus*, *Campylobacter*, *Pseudomonas*, and *Salmonella* are pathogenic for economically important livestock. Using the methods according to the present
15 invention, as illustrated in the following examples, *htrB* mutants of such animal pathogens can be produced. The resultant *htrB* mutants, or endotoxin isolated therefrom, can be used in a vaccine formulation. Use of vaccine formulations, containing one or more antigens of
20 various microbial pathogens, in animal feed has been described previously (See for example, Pritchard et al., 1978, *Avian Dis* 22:562-575).

EXAMPLE 5

25 *H. influenzae htrB* mutants as immunogens

In one embodiment, the *htrB* mutant is an *H. influenzae htrB* mutant. *Haemophilus influenzae* is an important human respiratory tract pathogen in diseases including otitis media, chronic sinusitis, and chronic
30 obstructive pulmonary disease. Certain surface-exposed bacterial components, including P2, P6, and LOS, appear to be antigens which may confer a protective immune response in immunized humans. Such antigens have been shown to be targets of bactericidal antibody, and the
35 presence of serum bactericidal antibody is associated

with protection from infection by *H. influenzae* (Faden et al., 1989, *J. Infect. Dis.* 160:999-1004).

5.1 In one aspect of the this embodiment, the endotoxin isolated from an *htrB* mutant is used as the immunogen in a vaccine formulation. As demonstrated in Example 3 herein, the endotoxin isolated from an *htrB* mutant of a gram-negative bacterial pathogen lacks one or more secondary acyl chains, and thus exhibits substantially reduced toxicity as compared to endotoxin isolated from the respective wild type bacterial pathogen. Therefore, endotoxin isolated from an *htrB* mutant of *H. influenzae* can be used in a vaccine formulation in inducing immunity against the respective wild type strain. The *htrB* mutant LOS may be isolated by a method known to those skilled in the art for isolating LOS. The *htrB* mutant LOS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

To illustrate the effects of immunization with *htrB* mutant LOS, a mouse model was used. NTHi 2019 LOS, and *htrB* mutant NTHi B29 LOS were each isolated from their respective strains using the phenol-water method. Groups of at least 5 Swiss Webster mice were immunized subcutaneously with 1 μ g of either NTHi 2019, *htrB* mutant B29 LOS, or *htrB* mutant conjugated to a carrier protein, with adjuvant QS-21. Sera was collected from each group 5 weeks after immunization, and the sera from animals comprising a group were pooled. The pooled sera was assessed for titer of anti-LOS antibody by enzyme-linked immunosorbent assay (ELISA). Microtiter wells of ELISA plates were coated with either 10 μ g of NTHi 2019, or *htrB* mutant B29 LOS. FIG. 5 illustrates the mean titers of anti-LOS antibody against NTHi 2019 LOS (antigen coating) in ELISA from mice immunized with NTHi.

2019 (Pool 595), *htrB* mutant B29 LOS (Pool 597), or *htrB* mutant B29 LOS conjugated to a carrier protein (Pool 606). FIG. 6 illustrates the mean titers of anti-LOS antibody against *htrB* mutant B29 LOS (antigen coating)
5 in ELISA from mice immunized with NTHi 2019 (Pool 595), *htrB* mutant B29 LOS (Pool 597), or *htrB* mutant B29 LOS conjugated to a carrier protein (Pool 606). Pre-immune sera was also included as a control ("week 0" or "time 0").

10 The antibody induced by the different LOS preparations were then tested for functional activity by performing bactericidal assays using NTHi 2019 as the target. Into the wells of a 96-well plate are added 0.150 ml buffer, 0.040 ml pooled human sera as a
15 complement source, and 0.01ml of NTHi 2019. Typically, the organism is plated in a dilution (e.g. 20 to 200 cfu). Into test wells are added the respective antisera either undiluted ("neat"), 1/10 dilution, or 1/100 dilution. The plate is then rotated vigorously (175-200
20 rpm) at 37°C for 30 minutes. Aliquots from respective wells are plated on media and grown to determine percentage survival, and log kill. Log kill is calculated as the log(cfu 30 minutes/cfu time 0). The results of the bactericidal assay are shown in Table 1,
25 where Group R595 is the antisera induced by NTHi 2019 LOS, Group R597 is the antisera induced by *htrB* mutant B29 LOS, and Group R606 is the antisera induced by *htrB* mutant B29 LOS conjugated to a carrier protein.

Table 1

30

Group	week	dilution	% survival	log kill
R595	0	neat	1.00	-1.99
		1/10	37.30	-0.43
		1/100	87.20	-0.06
	5	neat	-	-4.49
35		1/10	0.07	-3.15

		1/100	20.20	-0.70
	7	1/10	-	-4.62
		1/100	7.80	-1.11
5	R597	0	neat	3.50
				-1.46
		1/10	62.40	-0.20
		1/100	108.60	0.04
	5	neat	-	-4.49
		1/10	4.40	-1.36
		1/100	82.40	-0.08
10		7	1/10	-
				-4.49
		1/100	99.80	0.00
	R606	0	neat	0.13/110.5
				-2.87/0.92
		1/10	130.20	0.11
		1/100	106.50	0.03
15		5	neat	1.5/0.1
				-1.81/-2.9
		1/10	25.10	-0.60
		1/100	164.10	0.22
		7	1/10	0.04
				-3.44
20			1/100	185.90
				0.27

The greater the log kill (the more negative the number, e.g. -4.49), the greater the bactericidal activity is of the respective antibody. Thus, for comparison purposes, at week 7 and at a 1/10 dilution, log kill for the antisera induced by NTHi 2019 LOS is -4.62, the log kill for the antisera induced by NTHi *htrB* mutant B29 LOS is -4.49, and the log kill of the antisera induced by *htrB* mutant B29 LOS conjugated to a carrier protein is -3.44. By ELISA, it looks like the antisera induced by *htrB* mutant B29 LOS antibody is low in titer; yet, as demonstrated by the bactericidal assays, significant functional antibody is raised by immunization with *htrB* mutant B29 LOS.

5.2 In another aspect of the this embodiment, *htrB* mutant bacterial cells are used as the immunogen in a vaccine formulation. To illustrate the effects of immunization with *htrB* mutant bacterial cells, an infant rat model was used. The use of the infant rat model as a model of bacteremic infections due to type b *H. influenzae* (Hib) in humans, and for determining the virulence of type b *H. influenzae* strains, has been accepted by those skilled in the art (see, e.g., Smith et al., 1973, *Infect. Immun.* 8:278-290; Moxon et al., 1974, *J. Infect. Dis.* 129:154-62; Rubin et al., 1983, *Infect. Immun.* 41:280-284; Zwahlen et al., 1985, *J. Infect. Dis.* 152:485-492).

Type b strain A2 of *H. influenzae* has already been characterized as a highly virulent strain in the infant rat model system that causes bacteremia and meningitis after inoculation (e.g., intraperitoneal or intranasal), and as a clinical isolate from humans (it isolated from a child with meningitis due to *H. influenzae*). Using the methods according to Example 1, an *htrB* mutant was made from Hib strain A2. One week old infant Sprague-Dawley albino rats were inoculated intraperitoneally with either 10^7 Hib strain A2 or 10^7 *htrB* A2 mutant and then assessed for intravascular clearance by measuring the number of colony forming units (cfus) per ml of blood obtained 48 hours post-inoculation. The results showed that 20 of 20 infant rats inoculated with Hib strain A2 showed bacteremia, with all rats showing greater than 10^5 cfu/ml of strain A2. In contrast, only 13 of 20 infant rats inoculated with *htrB* A2 mutant showed bacteremia, with only 10 of the 13 showing greater than 10^5 cfu/ml of *htrB* A2 mutant.

Similarly, one week old infant Sprague-Dawley albino rats were inoculated intranasally with either 10^7 Hib strain A2 or 10^7 *htrB* A2 mutant and then assessed for intravascular clearance. The results showed that 8 of

20 infant rats inoculated with Hib strain A2 showed bacteremia, with 7 of those 8 rats showing greater than 10^5 cfu/ml of strain A2. In contrast, none of the 30 infant rats inoculated with *htrB* A2 mutant showed bacteremia. Taken together, it can be concluded from this model system that *htrB* mutants demonstrate attenuated virulence, as compared to its wild-type strain, as indicated by the decreased ability to cause bacteremia (e.g., a 30% reduction in the occurrence of bacteremia).

To further illustrate the effects of immunization with *htrB* mutant bacterial cells, a chinchilla model was used. The use of the chinchilla model as a model of middle ear infections due to nontypable *H. influenzae* (NTHi) in humans, and for determining the virulence of NTHi strains, has been accepted by those skilled in the art (see, e.g., Bakaletz et al., 1989, *Acta Otolaryngol.* 107:235-243; Madore et al., 1990, *Pediatrics* 86:527-34; Barenkamp, 1986, *Infect. Immun.* 52:572-78; Green et al., 1994, *Methods Enzymol.* 235:59-68).

NTHi 2019 is a clinical isolate described previously (see, e.g., Murphy et al., 1986, *Infect. Immun.* 54:774-779). Each healthy adult chinchilla was inoculated, via the epitympanic bulla into the middle ear space, with various log doses of either NTHi 2019 or *htrB* mutant NTHi B29. The course of middle ear disease was then assessed by periodic otoscopic examination for tympanic membrane inflammation or middle ear infusion, and aspiration from the middle ear with subsequent culture. The results showed that when compared to NTHi 2019, it takes up to a 3 log greater dose of *htrB* mutant NTHi B29 (10^7 cfu/ear) to induce middle ear infection. It can be concluded from this model system that *htrB* mutants demonstrate attenuated virulence, as compared to

its wild-type strain, as indicated by the decreased ability to cause middle ear disease.

In another aspect of this embodiment the *H. influenzae htrB* mutant is genetically engineered to express one or more heterologous bacterial antigens. As will be discussed in more detail below, *H. influenzae* has a natural genetic transformation process involving linearized DNA binding, uptake via one or more uptake sequences (e.g. AAGTGCGGT -SEQ ID NO:3), translocation, and recombination. Thus, one mechanism to introduce a recombinant DNA molecule containing the at least one heterologous bacterial antigen to be expressed, is to transform the host *H. influenzae htrB* mutant with linearized recombinant DNA molecule containing the DNA encoding the at least one heterologous bacterial antigen ("the encoding sequence"). Alternatively, the recombinant DNA molecule containing the encoding sequence to be expressed can be inserted into a plasmid vector, and either introduced into as a linearized recombinant molecule by the natural transformation process; as circularized recombinant plasmid using electroporation of noncompetent *H. influenzae htrB* mutants; or as a circularized recombinant plasmid transformed into competent *H. influenzae htrB* mutants.

Plasmids useful for cloning of and expression from recombinant DNA molecules in *H. influenzae* are known to those skilled in the art. Such plasmids include:

pRSF0885 confers ampicillin resistance, and contains a *PvuII* cloning site and a defective *TnA* sequence (Setlow et al., 1981, *J. Bacteriol.* 148:804-811), and can replicate in both *H. influenzae* and *E.coli* (Trieu et al., 1990, *Gene* 86:99-102).

pDM2 was constructed by cloning chloramphenicol resistance into **pRSF0885**; and **pDM5** was constructed by

cloning tetracycline resistance into pRSF0885 (McCarthy et al., 1986, *J. Bacteriol.* 168:186-191).

pVT63, pVT64, pVT65, pVT66 are improved shuttle vectors for *H. influenzae* and *E. coli* based on pDM2 (Trieu et al., 1990, *Gene* 86:99-102), and contain the pUC-derivative of the ColE1 ori, and the pRSF0885 rep locus. Additionally, each plasmid has drug markers with unique restriction sites for insertional inactivation of the drug marker as follows: pVT63- ApR (*HincII*, *PstI*, *ScaI*), KmR (*ClaI*, *HindIII*, *NruI*, *SmaI*, *XhoI*); pVT64- ApR (*HincII*, *PstI*, *ScaI*, *SspI*), SpR; pVT65- ApR (*HincII*, *PstI*, *ScaI*, *PvuI*, *SspI*), CmR (*BalI*, *NcoI*); pVT66- ApR (*HincII*, *PstI*, *ScaI*, *PvuI*), CmR (*SmaI*).

pACYC177, pACYC184, pSU2718, pSU2719 are improved shuttle vectors for *H. influenzae* and *E. coli* based on p15A (Chandler, 1991, *Plasmid* 25:221-224), have the p15A ori, and were compatible with a plasmid containing the RSF0885 origin of replication. Additionally, each plasmid has multiple cloning sites restriction sites and drug markers as follows: pACYC177- ApR, KmR (Accession No. X06402); pACYC184- CmR, TcR (Accession No. X06403); pSU2718- CmR and polycloning site from pUC18 (Accession No. M64731); and pSU2719- CmR and polycloning site from pUC19 (Accession No. M64732).

pQL1 is an improved shuttle vector for use in *H. influenzae* and *E. coli* containing both the pMB1 ori and P15a ori, KmR which is flanked by *H. influenzae* uptake sequences, a multiple cloning site containing a unique *BamHI* and *SmaI* restriction sites, and which is particularly suited for analyzing *H. influenzae* promoter strength in *H. influenzae* (Heidecker et al., 1994, *Gene* 150:141-144).

In cloning the recombinant DNA molecule containing the encoding sequence into a plasmid vector, one skilled in the art will appreciate that the choice of restriction enzymes for digesting both the

recombinant DNA molecule and the plasmid to result in compatible ends for ligation depends on the unique restriction enzyme sites at the ends of the recombinant DNA molecule, whether occurring naturally or engineered such as during enzymatic amplification; one or more unique restriction enzyme sites within the plasmid vector; whether insertion into the plasmid vector will assist in the selection process (See, e.g., pVT66); and whether a plasmid-derived promoter is used solely, or in addition to the promoter(s) of the encoding sequences, to drive expression from the recombinant DNA molecule. Selection and screening of transformed *H. influenzae* *htrB* mutants may be accomplished by methods known in the art including detecting the expression of a marker gene (e.g., drug resistance marker) present in the plasmid, and immunodetection of the expressed and displayed heterologous bacterial antigen. While this aspect of the embodiment illustrates that the recombinant DNA molecule containing the encoding sequence can be inserted into a plasmid and expressed in *H. influenzae* *htrB* mutants, it will be appreciated by those skilled in the art that vectors other than plasmids, can be used including, but not limited to, bacteriophage vectors.

Successful expression of the at least one heterologous bacterial antigen requires that either the recombinant DNA molecule comprising the encoding sequence, or the vector itself, contain the necessary control elements for transcription and translation which is compatible with, and recognized by the particular host system used for expression. Using methods known in the art of molecular biology, including methods described above, various promoters and enhancers can be incorporated into the vector or the recombinant DNA molecule containing the encoding sequence to increase the expression of the heterologous bacterial antigen, provided that the increased expression of the

heterologous bacterial antigen(s) is compatible with (for example, non-toxic to) the *htrB* mutant. As referred to herein, the encoding sequence can contain DNA encoding more than one heterologous bacterial antigen, and may include viral and/or fungal antigen-encoding sequences, to create a multivalent antigen for use as an improved vaccine composition.

The selection of the promoter will depend on the expression system used. For example, a preferred promoter in an *H. influenzae* expression system may be the P2 or P6 promoter operatively linked to the encoding sequence. Promoters vary in strength, i.e. ability to facilitate transcription. Generally, for the purpose of expressing a cloned gene, it is desirable to use a strong promoter in order to obtain a high level of transcription of the gene and expression into gene product. For example, bacterial, phage, or plasmid promoters known in the art from which a high level of transcription has been observed in a host cell system comprising *E. coli* include the lac promoter, trp promoter, recA promoter, ribosomal RNA promoter, the P_R and P_L promoters, lacUV5, ompF, bla, lpp, and the like, may be used to provide transcription of the inserted encoding sequence.

Other control elements for efficient gene transcription or message translation include enhancers, and regulatory signals. Enhancer sequences are DNA elements that appear to increase transcriptional efficiency in a manner relatively independent of their position and orientation with respect to a nearby gene. Thus, depending on the host cell expression vector system used, an enhancer may be placed either upstream or downstream from the encoding sequence to increase transcriptional efficiency. These or other regulatory sites, such as transcription or translation initiation signals, can be used to regulate the expression of the

encoding sequence. Such regulatory elements may be inserted into the recombinant DNA molecule containing the encoding sequence, or nearby vector DNA sequences using recombinant DNA methods described herein, and
5 known to those skilled in the art, for insertion of DNA sequences.

Accordingly, a recombinant DNA molecule containing an encoding sequence, can be ligated into an expression vector at a specific site in relation to the
10 vector's promoter, control, and regulatory elements so that when the recombinant vector is introduced into the *htrB* mutant, the heterologous bacterial antigen can be expressed in the host cell. The recombinant vector is then introduced into the *htrB* mutant, and the
15 transformed *htrB* mutants are selected, and screened for those cells containing the recombinant vector. Selection and screening may be accomplished by methods known in the art, and depending on the vector and expression system used.

20 The introduction of a recombinant DNA molecule containing the encoding sequence (including an expression vector or plasmid containing the same) into *H. influenzae htrB* mutants can be accomplished in any one of three processes: a natural genetic transformation
25 process; transformation of competent bacterial cells; and electroporation of non-competent bacterial cells.

Natural transformation process

The natural genetic transformation process of
30 *H. influenzae* involves linearized DNA binding, uptake via one or more uptake sequences, translocation, and recombination. Thus, one mechanism to introduce a recombinant DNA molecule containing the encoding sequence to be expressed into at least one heterologous
35 bacterial antigen, is to transform the host *H. influenzae* with linearized recombinant DNA molecule

containing the encoding sequence; or a linearized vector having inserted into it the recombinant DNA molecule containing the encoding sequence to be expressed. In this natural process, when the linearized DNA is translocated intracellularly, one of the translocated strands of DNA is apparently degraded by exonuclease activity (Barany et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:7274-7278). If the translocated strand lacks homology sufficient for recombination into the *H. influenzae* chromosome, the translocated strand becomes susceptible to further degradation (Pifer et al., 1985, *Proc. Natl. Acad. Sci. USA* 82:3731-3735). Using methods known in the art (e.g., Barany et al., 1983, *supra*; herein incorporated by reference), linearized DNA containing the encoding sequence can be introduced into *H. influenzae htrB* mutants. Since the encoding sequence can be flanked by *H. influenzae* sequences, to increase the likelihood of recombination of the encoding sequence into the *H. influenzae htrB* mutants' genome is likely to occur.

Transformation of competent bacterial cells

Another mechanism to introduce a recombinant DNA molecule containing the encoding sequence to be expressed into at least one heterologous bacterial antigen, is to transform competent host *H. influenzae htrB* mutants with a circular vector, such as a plasmid, having inserted into it the recombinant DNA molecule containing the encoding sequence to be expressed. Competence of *H. influenzae* develops best under conditions in which the bacterial cell duplication is inhibited, such as a temporary shift to anaerobic conditions, by physiological change occurring during late-log phase growth, and transfer of cells into nutrient-poor, chemically-defined medium. Such defined media for the development of competence of *H. influenzae*.

has been previously described in detail (Herriott et al., 1970, *J. Bacteriol.* 101:517-524; herein incorporated by reference). It appears that only a short time after entering competent *H. influenzae*, a
5 plasmid containing sequences homologous to the bacterial chromosome can insert its homologous sequence (such as the encoding sequence flanked by *H. influenzae* sequences) into the chromosome via recombination (Setlow et al., 1981, *supra*). for expression. Thus, in this
10 embodiment, a plasmid containing the encoding sequence which is capable of being transformed into competent *H. influenzae htrB* mutants is introduced by methods for transformation known in the art (Karudapuram et al., 1995, *J. Bacteriol.* 177:3235-3240; Setlow et al., 1981,
15 *supra*, herein incorporated by reference). The encoding sequence may then be recombined into the *H. influenzae htrB* mutants' genome where it is expressed under the control of its own promoter or an *H. influenzae* promoter near the site of insertion. Such transformation is
20 reported to be at a relatively high frequency (McCarthy and Cox, 1986, *J. Bacteriol.*, 168:186-191).

Alternatively, transformation of competent *H. influenzae htrB* mutants by a circular plasmid with the appropriate origin(s) of replication and containing the
25 encoding sequence may result in plasmid establishment; i.e., a plasmid coexisting as an extrachromosomal element without recombination. Examples of such plasmids have been described above. Thus, in this variation of the embodiment, a plasmid containing the
30 encoding sequence which is capable of being transformed into, and established in, competent *H. influenzae htrB* mutants is introduced by methods for transformation known in the art. The encoding sequence is then expressed from the plasmid under the control of its own
35 promoter or a promoter within the vector.

Electroporation of non-competent bacterial cells

Yet another mechanism to introduce a recombinant DNA molecule containing the encoding sequence to be expressed into at least one heterologous bacterial antigens, is to introduce a circular vector, such as a plasmid having inserted into it the recombinant DNA molecule containing the encoding sequence to be expressed, into non-competent host *H. influenzae htrB* mutants by electroporation.

Electroporation has been used to efficiently introduce plasmid DNA into bacteria. However, optimal conditions may differ depending on the host cell used. Optimal conditions have been described for electroporating plasmid DNA into *H. influenzae* (Mitchell et al., 1991, *Nucl. Acids Res.* 19:3625-3628; herein incorporated by reference). It was found that electroporation of plasmid into *H. influenzae* made competent by defined, nutrient poor media was several orders of magnitude less efficient than electroporation into non-competent *H. influenzae*. Thus, in this variation of the embodiment, it would be preferred that a plasmid containing the encoding sequence is electroporated into non-competent *H. influenzae htrB* mutants. The plasmid is capable of being established in *H. influenzae htrB* mutants, or is degraded after the encoding sequence has recombined into the *H. influenzae htrB* mutants' genome. In either case, the encoding sequence is under the control of its own promoter; or a promoter within the vector or genome, respectively.

30

EXAMPLE 6

Neisserial htrB mutants as immunogens

In another embodiment, the *htrB* mutant is a *Neisserial htrB* mutant selected from the group including *Neisseria gonorrhoeae*, and *Neisseria meningitidis*. *N. gonorrhoeae* is a gram-negative bacterial pathogen

35

causing the sexually transmitted disease gonorrhea, which subsequently can lead to pelvic inflammatory disease in females. *N. meningitidis* is a gram-negative bacterial pathogen which can cause a variety of clinical
5 infections including bacteremia, septicemia, meningitis, and pneumonia. Alterations in the terminal glycosylation of the LOS of *Neisseria* are believed correlate with serum sensitivity and serum resistance of the organism. Further, protective bactericidal antibody
10 is directed against type-specific antigens of *N. meningitidis*, wherein the type-specific antigens have been identified as outer membrane proteins, or LOS, or both.

Using the methods according to the present
15 invention, as illustrated in Examples 1-3 and 10, *htrB* mutants of a *Neisserial* species can be produced and identified. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of the *Neisserial* species, and produce a mutated *htrB* gene
20 (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in a *Neisserial htrB* mutant lacking one or more secondary acyl chains. Alternatively, there may be sufficient homology between *Neisseria* and *Haemophilus* to use
25 plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform the *Neisserial* species for recombination of the mutant *htrB* gene into the
30 *Neisserial htrB* gene. *Neisserial* transformants are then selected for by growth in the presence of chloramphenicol (1.5 µg/ml), resulting in identification of *Neisserial htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *Neisserial htrB*
35 mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences.

The resultant *Neisserial htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

5 Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *Neisserial htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *Neisserial* pathogens. The *htrB*
10 mutant LOS may be isolated by a method known to those skilled in the art for isolating LOS. The *htrB* mutant LOS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a
15 physiological solution), an adjuvant, or a carrier protein.

 Alternatively, *Neisserial htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be
20 genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *Neisserial* species are known to those
25 skilled in the art, including: pLES2 confers ampicillin resistance, is a shuttle vector functional in both *E. coli* and *N. gonorrhoeae*, and contains a polylinker with restriction sites for *EcoRI*, *SmaI*, and *BamHI* (Stein et al., 1983, *Gene* 25:241-247).
30 *Neisserial* species also contain a natural transformation process (Rudel et al., 1995, *Proc Natl Acad Sci USA* 92:7896-90; Goodman et al., 1991, *J Bacteriol* 173:5921-5923); and can also be made competent or be
35 electroporated using techniques known to those skilled in the art.

EXAMPLE 7

Haemophilus ducreyi htrB mutants as immunogens

In another embodiment, the mutant is a *H. ducreyi htrB* mutant. *H. ducreyi* is a gram-negative bacterial pathogen causing a genital ulcer disease, chancroid. Using the methods according to the present invention, as illustrated in Examples 1-3 and 10, *H. ducreyi htrB* mutants can be produced and identified. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *H. ducreyi*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in an *H. ducreyi htrB* mutant lacking one or more secondary acyl chains. Alternatively, there is likely sufficient homology between *H. ducreyi* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *H. ducreyi* for recombination of the mutant *htrB* gene into the *H. ducreyi htrB* gene. *H. ducreyi* transformants are then selected for by growth in the presence of chloramphenicol (1.5 µg/ml), resulting in identification of *H. ducreyi htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *H. ducreyi htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *H. ducreyi htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from an *H. ducreyi htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *H. ducreyi*. The *htrB* mutant LOS

may be isolated by a method known to those skilled in the art for isolating LOS. The *htrB* mutant LOS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a
5 pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *H. ducreyi htrB* mutants can be used in a live bacterial vaccine preparation, in an
10 inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant
15 DNA molecules into *Haemophilus* species are known to those skilled in the art, as disclosed in Example 5; and can also be made competent or be electroporated using techniques known to those skilled in the art, as disclosed in Example 5.

20

EXAMPLE 8

Campylobacter jejuni htrB mutants as immunogens

In another embodiment, the mutant is a *C. jejuni htrB* mutant. *Campylobacter jejuni* is a gram-
25 negative bacterial pathogen causing human enteritis. Infection by *C. jejuni* has also been associated with the onset of neurologic disorders such as Guillian-Barré syndrome. *C. jejuni htrB* mutants can be produced and identified using the methods according to the present
30 invention, as illustrated in Examples 1-3 and 10. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *C. jejuni*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent
35 recombination resulting in an *C. jejuni htrB* mutant lacking one or more secondary acyl chains.

Alternatively, there may be sufficient homology between *C. jejuni* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *C. jejuni* for recombination of the mutant *htrB* gene into the *C. jejuni htrB* gene. *C. jejuni* transformants are then selected for by growth in the presence of chloramphenicol (1.5 µg/ml), resulting in identification of *C. jejuni htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *C. jejuni htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *C. jejuni htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *C. jejuni htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *C. jejuni*. The *htrB* mutant LPS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant LPS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *C. jejuni htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant

DNA molecules into *C. jejuni* are known to those skilled in the art, and includes:

pUA466 confers tetracycline resistance, and contains an unique *Ava*I site and *Ava*II site (Taylor, 1986, *J*

5 *Bacteriol* 165:1037-39).

C. jejuni can also be made competent or be electroporated using techniques known to those skilled in the art.

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EXAMPLE 9

Moraxella catarrhalis htrB mutants as immunogens

In another embodiment, the mutant is a *M. catarrhalis htrB* mutant. *Moraxella catarrhalis* is a gram-negative bacterial pathogen causing otitis media in
15 children; sinusitis and conjunctivitis in children and adults; and lower respiratory tract infections, septicemia, and meningitis in immunocompromised hosts. *M. catarrhalis htrB* mutants can be produced and identified using the methods according to the present
20 invention, as illustrated in Examples 1-3 and 10. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *M. catarrhalis*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with
25 subsequent recombination resulting in an *M. catarrhalis htrB* mutant lacking one or more secondary acyl chains. Alternatively, there may be sufficient homology between *M. catarrhalis* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the
30 chloramphenicol acetyltransferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *M. catarrhalis* for recombination of the mutant *htrB* gene into the *M. catarrhalis htrB* gene. *M. catarrhalis* transformants are then selected
35 for by growth in the presence of chloramphenicol (1.5 µg/ml), resulting in identification of *M. catarrhalis*

htrB mutant strains. Locations of the mTn3 insertion in the chromosomes of the *M. catarrhalis htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *M.*

5 *catarrhalis htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *M. catarrhalis htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *M. catarrhalis*. The *htrB* mutant LOS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant LOS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *M. catarrhalis htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *M. catarrhalis* are known to those skilled in the art. *M. catarrhalis* contains a natural transformation process (Juni, 1977, *J Clin Microbiol* 5:227-35) and can also be made competent or be electroporated using techniques known to those skilled in the art.

EXAMPLE 10

35 *Salmonella htrB* mutants as immunogens

In another embodiment, the mutant is a *Salmonella htrB* mutant. *Salmonella* species comprise gram-negative bacteria that can cause a variety of clinical illnesses in humans and animals. For example, *S. typhi* is the causative agent of typhoid fever in humans. *S. paratyphi* is a causative organism of a fever known as salmonella fever in humans. Salmonellosis, a gastroenteritis in humans, can be caused by various species in the genus *Salmonella* (*typhimurium*, *newport*, *heidelberg*, and *enteritidis*). *Salmonella htrB* mutants can be produced and identified. One skilled in the art, using the *htrB* gene of a gram-negative bacterial pathogen, can produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis and subsequent recombination, ultimately resulting in an *Salmonella htrB* mutant having a modification in one or more secondary acyl chains. The resultant *Salmonella htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled in the art for measuring the toxic effects induced by endotoxin.

To illustrate this embodiment, and using methods similar to those in Example 1 herein, mutagenesis of the *htrB* gene was carried out by shuttle mutagenesis by mini-Tn10 (conferring tetracycline resistance) used as an insertion sequence to mutate the *htrB* gene. The *htrB*:Tn10 was then transferred from *E. coli* to a virulent *S. typhimurium* by transduction. Using methods previously described (Masters, 1996, in *E. coli and Salmonella Cellular and Molecular Biology*, vol.2, 2nd edition, p. 2421, ASM Press), a *rm⁺ galE mutS recD* *S. typhimurium* (SL5283) was sequentially transduced with MST3488 (*recD542*:Tn10d which confers chloramphenicol resistance (*cm^r*)) via *Salmonella* phage P22 resulting in a *rm⁺ galE mutS recD cm^r S. typhimurium* ("MGS-1"), and then with MST3063 (*mutS*:Tn10 which

confers tetracycline resistant (tet^r) resulting in a r^m
m⁺ galE mutS recD cm^r tet^r *S. typhimurium* ("MGS-3"). *S.*
typhimurium MGS-3 was cured of Tn10 by selection for
tetracycline sensitivity on media using methods
5 previously described (Bochner et al., 1990, *J.*
Bacteriol. 143:926-933) resulting in a r^m⁺ galE mutS
recD cm^r *S. typhimurium* ("MGS-7"). For confirmation
purposes, it was shown that *S. typhimurium* MGS-7 showed
the same response to ultraviolet light as the parental
10 strain MGS-3.

An *E. coli* strain ("MLK217") containing the
htrB:mini Tn10 was used to transfer the *htrB*:Tn10 by
transduction into *S. typhimurium* MGS-7 via coliphage P1
according to methods previously described (Masters,
15 1996, *supra*), and selected for by growth at 30°C on
media plates containing tetracycline. The result of the
transduction, and after reisolation for tetracycline-
resistant clones, was the creation of a r^m⁺ galE mutS
recD *htrB*:mini Tn10 cm^r tet^r *S. typhimurium* ("MGS-23").
20 *S. typhimurium* MGS-23 was tested for one or more of the
phenotypic properties associated with *htrB* mutation,
namely (1) temperature sensitivity; (2) filamentation
and bulging at non-permissive temperatures; and (3)
deoxycholate resistance. The results of the phenotypic
25 analysis indicated that MGS-23 carried the miniTn 10
element inserted within the *S. typhimurium htrB* gene
because MGS-23 was able to grow at 30°C but not at 37°C;
formed many filamentous forms when shifted to non-
permissive temperature; and showed resistance to higher
30 concentrations of deoxycholate (7.5% to 10%) than the
isogenic parent (2.5%). The mutation of the *htrB* was
further confirmed by analysis using polymerase chain
reaction.

A virulent *S. typhimurium* strain (SL1344) was
35 transduced to *htrB*:Tn10 from *S. typhimurium* MGS-23 via
Salmonella phage P22 and selection at 30°C on media

plates containing tetracycline. After isolation, resultant tetracycline resistant clones having the same phenotype as MGS-23 were further analyzed. One such clone, MGS-31, was shown to have a mutated *htrB* gene, by
5 complementing the clone using a plasmid with a wild type *htrB* gene (Karow et al., 1991, *J. Bacteriol.* 173:741-50; Karow et al., 1991, *Mol. Microbiol.* 5:2285-2292) thereby returning the clone to the wild type phenotype of normal growth, normal cell morphology, deoxycholate sensitivity
10 at 37°C, and wild type virulence.

Endotoxin characteristics

Mass spectrometry was used to analyze the lipid A according to the methods in Example 2 herein. More specifically, lipid A from *S. typhimurium htrB*
15 mutant LPS and from wild type *S. typhimurium* LPS were each analyzed by liquid secondary ion mass spectrometry (LSIMS) in the negative ion mode to provide a spectrum of molecular ions for the different components lipid A. The chemical analysis of the lipid A of the *S.*
20 *typhimurium htrB* mutant indicated that the modifications in the lipid A structure that occurred were similar, but not identical, to modifications in the lipid A structure seen in *H. influenzae htrB* mutants. In the wild type *S. typhimurium* lipid A contains either six (hexaacyl) or
25 seven (heptaacyl) fatty acid substitutions from the diglucosamine backbone (FIG. 7). In the wild type strain, on glucosamine II, the 3' substitution on the N-linked C14 fatty acid (hexaacyl or heptaacyl) is a C12 fatty acid. In contrast, and as shown in FIG. 7, the
30 C12 fatty acid is replaced with a C16 fatty acid. These results indicate that the *S. typhimurium htrB* gene encodes an acyltransferase responsible for placing the C12 fatty acid at the 3' position on the N-linked C14 fatty acid. Mutation of the *S. typhimurium htrB* gene
35 results in the functional induction of another acyltransferase which places a C16 fatty acid at the 3'

position on the N-linked C14 fatty acid. It is known to those skilled in the art, that lipid A is crucial for the survival of a gram-negative organism, and for the proper organization of its outer membrane. Thus, and as
5 related to virulence and toxicity of the organism, the effects of the *htrB* gene mutation in *S. typhimurium* was analyzed.

Endotoxin toxicity

A mouse model system that is used by those
10 skilled in the art as relevant to human disease, is the D-galactosamine model. In the D-galactosamine model, the sensitivity to LPS is increased by exposure to D-galactosamine (Galanos et al., 1986, *Infect. Immun.* 51:891-896) thereby achieving the same lethality and TNF
15 induction with low doses of LPS, i.e. with levels of endotoxin commensurate with those identified in the blood of septic patients. Thus, D-galactosamine-treated mice exposed to LPS is a standard animal model system accepted by those skilled in the art as relevant to
20 endotoxic shock in humans. In this model, groups of 4 mice were treated with D-galactosamine (8 mg) simultaneously with the administration of the dosage of the LPS to be tested. Groups of 4 mice each were
25 injected with either 0.001 μ g, 0.01 μ g, 0.1 μ g, 1 μ g or 10 μ g of the purified LPS to be tested and the number of mice surviving the challenge at each dose were checked every 24 hours for 5 days after the injection. The LD₅₀ (lethal dose where 50% of the mice are killed) is then
30 calculated. The purified LPS to be separately tested included LPS from the wild type virulent *S. typhimurium* strain 1344; and the *S. typhimurium htrB* mutant (MGS-31). The results show that the LD₅₀ for mice injected with LPS from *S. typhimurium* strain 1344 is 0.01 μ g. In contrast, the LD₅₀ for mice injected with LPS from *S.*
35 *typhimurium htrB* mutant MGS-31 is 0.1 μ g. Thus, the lipid A from the *S. typhimurium htrB* mutant is at least

10 fold less toxic than the lipid A of the wild type strain.

Virulence

Since mice are naturally susceptible to infection by *S. typhimurium*, like humans, a second mouse model was used to assess the effects of the *htrB* mutation on virulence of *S. typhimurium*. Typically, from approximately 50 cfu to 100 cfu will kill 50 to 100% of the mice injected. The strains used included *S. typhimurium* strain 1344; *S. typhimurium htrB* mutant (MGS-31); and the *S. typhimurium htrB* mutant which was complemented by the plasmid containing an intact *htrB* gene (MGS-43). The respective organisms were injected intraperitoneally in a dosage ranging from 5×10^1 to 5×10^7 cfu and watched over 5 days. Generally, 100% of the animals who will die from bacteremia, do so within that period. As may be expected, the LD_{50} for the virulent *S. typhimurium* strain 1344 was less than 5×10^1 cfu. Likewise, the LD_{50} for the *S. typhimurium htrB* mutant which was complemented with the intact *htrB* gene, MGS-43, also was less than 5×10^1 cfu. In contrast, the LD_{50} for the *S. typhimurium htrB* mutant MGS-31 was 9.7×10^6 cfu, an approximately 2×10^5 reduction in virulence compared to the wild type virulent strain. *S. typhimurium htrB* mutant growth in vivo in mice was confirmed by culturing and assaying liver and spleen for bacterial counts. The results suggest that the *htrB* mutation in *Salmonella* has a more profound effect on virulence factors than just a modification of the LPS.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from a *Salmonella htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of the *Salmonella* species. The *htrB* mutant LPS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant

LPS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *Salmonella htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *Salmonella* are known to those skilled in the art, and includes:

pYA260 containing *lacZ* cloned into a *trc* promoter; and pJW270 conferring tetracycline resistance and containing *lacI* (Ervin et al., 1993 *Microb Pathogen* 15:93-101). pB7 confers kanamycin and chloramphenicol resistance, and contains a cloning site flanked by a *BalI* site and a *HindIII* site (Purcell et al., 1983, *Infect Immun* 39:1122-1127).

pACK5 contains the replicon of pAC1 from *Acetobacter pasteurianus* and confers kanamycin resistance (Grones et al., 1995, *Biochem Biophys Res Commun* 206:942-947).

pVAC468 is a suicide vector for chromosomal insertion of heterologous antigens into *Salmonella* and contains a polylinker having the following restriction sites: *ClaI*, *EcoRV*, *XhoI*, *SacI*, *SalI*, *SmaI*, *XbaI*, and *BglII* (Hohmann et al., 1995, *Proc Natl Acad Sci USA* 92:2904-2908).

Also disclosed is a bacteriophage system, a 'chromosomal expression vector' for inserting genes encoding foreign antigens into the chromosome of *Salmonella*, which uses a defective transposable element carried on bacteriophage lambda (Flynn et al., 1990, *Mol Microb* 4:2111-2118).

Salmonella can also be made competent (see for example, Purcell et al., 1983, *supra*) or be electroporated using

techniques known to those skilled in the art (see for example, Gronos et al., 1995, *supra*; Coulson et al., 1994, *supra*).

EXAMPLE 11

5 *Shigella htrB* mutants as immunogens

 In another embodiment, the mutant is a *Shigella* species *htrB* mutant. Members of the genus *Shigella* are gram-negative bacteria which cause diseases such as dysentery (pathogenic species include
10 *dysenteriae*, *sonnei*, and *flexneri*) primarily in humans. *Shigella htrB* mutants can be produced and identified using the methods according to the present invention, as illustrated in Examples 1-3 and 10. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate
15 the *htrB* gene of *Shigella*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in an *Shigella htrB* mutant lacking one or more secondary acyl chains. Alternatively, there may be sufficient
20 homology between *Shigella* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyl-transferase (CAT) gene inserted into the *htrB* open reading frame at a different location, to transform *Shigella* for
25 recombination of the mutant *htrB* gene into the *Shigella htrB* gene. *Shigella* transformants are then selected for by growth in the presence of chloramphenicol (1.5 μ g/ml), resulting in identification of *Shigella htrB* mutant strains. Locations of the mTn3 insertion in the
30 chromosomes of the *Shigella htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences. The resultant *Shigella htrB* mutants can then be tested for substantially reduced toxicity using assays described by those skilled
35 in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin isolated from an *htrB* mutant made from a pathogenic *Shigella* species can be used in a vaccine formulation in inducing immunity against the wild type strains of *Shigella*. The *htrB* mutant LPS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant LPS may be used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

Alternatively, *Shigella htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *Shigella* are known to those skilled in the art, and includes: *pACK5* contains the replicon of *pAC1* from *Acetobacter pasteurianus* and confers kanamycin resistance (Grones et al., 1995, *supra*). *Shigella* can also be made competent or be electroporated using techniques known to those skilled in the art.

EXAMPLE 12

Pseudomonas aeruginosa htrB mutants as immunogens

In another embodiment, the mutant is a *Pseudomonas aeruginosa htrB* mutant. *Pseudomonas aeruginosa* is a gram-negative bacterial pathogen which cause diseases such as respiratory tract infections and sepsis, particularly in immunocompromised patients. Other pathogenic species for humans and animals include *pseudomallei*, and *mallei*. Mass spectrometry and nuclear

magnetic resonance spectroscopy were used to determine the structure of lipid A of *Pseudomonas aeruginosa* LPS. The structure of *P. aeruginosa* lipid A was found to be the same as *Enterobacterial* lipid A: a backbone of a
5 glucosamine disaccharide which is either mono-phosphorylated or diphosphorylated (positions 1 and 4'); and which carries several molecules of ester- and amide-bound fatty acids. In addition to the hexaacyl and pentaacyl lipid A species, a tetraacyl species was
10 identified (Karunaratne et al., 1992, Arch Biochem Biophys 299:368-76).

Pseudomonas htrB mutants (e.g., *P. aeruginosa*) can be produced and identified using the methods according to the present invention, as illustrated in
15 Examples 1-3 and 10. One skilled in the art, using the *htrB* gene of *H. influenzae*, can isolate the *htrB* gene of *Pseudomonas aeruginosa*, and produce a mutated *htrB* gene (unable to encode functional HtrB) using transposon mutagenesis with subsequent recombination resulting in a
20 *P. aeruginosa htrB* mutant lacking one or more secondary acyl chains. Alternatively, there may be sufficient homology between *P. aeruginosa* and *H. influenzae* to use plasmids pB28 and pB29, each with a mini-Tn3 transposon containing the chloramphenicol acetyltransferase (CAT)
25 gene inserted into the *htrB* open reading frame at a different location, to transform *P. aeruginosa* for recombination of the mutant *htrB* gene into the *P. aeruginosa htrB* gene. *P. aeruginosa* transformants are then selected for by growth in the presence of
30 chloramphenicol (1.5 µg/ml), resulting in identification of *P. aeruginosa htrB* mutant strains. Locations of the mTn3 insertion in the chromosomes of the *P. aeruginosa htrB* mutants may be confirmed by genomic Southern hybridization using a probe containing *htrB* sequences.
35 The resultant *P. aeruginosa htrB* mutants can then be tested for substantially reduced toxicity using assays

described by those skilled in the art for measuring the toxic effects induced by endotoxin.

Using the methods according to the present invention, as illustrated in Examples 4 & 5, endotoxin
5 isolated from a *P. aeruginosa htrB* mutant can be used in a vaccine formulation in inducing immunity against the wild type strains of *P. aeruginosa*. The *htrB* mutant LPS may be isolated by a method known to those skilled in the art for isolating LPS. The *htrB* mutant LPS may be
10 used in a vaccine formulation containing one or more agents selected from the group consisting of a pharmaceutically acceptable carrier (e.g., a physiological solution), an adjuvant, or a carrier protein.

15 Alternatively, *P. aeruginosa htrB* mutants can be used in a live bacterial vaccine preparation, in an inactivated bacterial vaccine preparation, and can be genetically engineered to express at least one heterologous bacterial antigen in a multivalent vaccine
20 preparation. Regarding the latter aspect, plasmids useful for cloning of and expression from recombinant DNA molecules into *P. aeruginosa* are known to those skilled in the art, and includes:

pPAH121 confers carbenicillin resistance, and contains a
25 unique *HpaI* restriction site (Hoyne et al., 1992, *J Bacteriol* 174:7321-7327.

P. aeruginosa can also be made competent (see for example, Hoyne et al., 1992, *supra*) or be electroporated using techniques known to those skilled in the art.

30

EXAMPLE 13

Multivalent *htrB* mutant vaccine formulation

In one embodiment according to the present invention, as illustrated in Examples 4 & 5, the *htrB*
35 mutant is genetically engineered to express one or more heterologous microbial antigens in producing a

multivalent vaccine using methods known to those skilled in the art. In a preferred embodiment, a microbial pathogen may include a respiratory pathogen selected from the group of pathogens, with respective antigens, in Table 2.

Table 2

	PATHOGEN	INFECTION/DISEASE	PROTEIN ANTIGEN
	<i>H. influenzae</i>	otitis media, lower respiratory tract	D-15, P1, P6 ¹
10	Group A <i>Streptococcus</i>	pharyngitis, rheumatic fever	M ²
	<i>Branhamella catarrhalis</i>	otitis media, lower respiratory tract	CD, E ³
15	<i>Streptococcus pneumoniae</i>	pneumonia, otitis media, meningitis	autolysin, pneumolysin ⁴
	<i>Bordetella pertussis</i>	pertussis (whooping cough)	filamentous hem-agglutinin, pertussis toxin, 69kDa Omp ⁵
	<i>Pseudomonas aeruginosa</i>	respiratory tract	Omp OprF, exotoxin A ⁶
20	<i>Legionella pneumophila</i>	pneumonia	OmpS, Hsp60 ⁷
	<i>Mycoplasma pneumoniae</i>	upper and lower respiratory tract	P1 ⁸
25	Respiratory syncytial virus	lower respiratory tract	M2, P, F, G ⁹
	Influenza virus	influenza	HA, M ¹⁰
	Adenovirus	common cold	
	rhinovirus	common cold	VP1, VP2, VP3 ¹¹
30	Parainfluenza virus	common cold	HN, F ¹²
	<i>Pneumocystis carinii</i>	pneumonia in AIDS	msg ¹³

- 1- (Flack et al., 1995 *Gene* 156:97-99; Panzutti et al., 1993, 61:1867-1872; Nelson et al., 1988, *Rev Infect Diseases* 10:S331-336).
- 2- (Pruksakorn et al., 1994, *Lancet* 344:639-642; Dole et al., 1993, *J Immunol* 151:2188-94).
- 3- (Murphy et al., 1989, *Infect Immun* 57:2938-2941; Faden et al., 1992, *Infect Immun* 60:3824-3829).
- 4- (Lock et al., 1992, *Microb Pathog* 12:137-143).
- 5- (Novotny et al., 1991, *Dev Biol Stand* 73:243-249; Lipscombe et al., 1991, *Mol Microbiol* 5:1385-1392; He et al., 1993, *Eur J Clin Microbiol Infect Dis* 12:690-695).
- 6- (Rawling et al., 1995, *Infect Immun* 63:38-42; Pennington et al., 1988, *J Hosp Infect* 11A:96-102).
- 7- (Weeratna et al., 1994, *Infect Immun* 62:3454-3462).
- 8- (Jacobs et al., 1990, *Infect Immun* 58:2464-2469; 1990, *J Clin Microbiol* 28:1194-1197).
- 9- (Kulkarni et al., 1995, *J Virol* 69:1261-1264; Leonov et al., 1994, *J Gen Virol* 75:1353-1359; Garcia et al., 1993, *Virology* 195:239-242; Vaux-Peretz et al., 1992, *Vaccine* 10:113-118).
- 10- (Kaly et al., 1994, *Vaccine* 12:753-760; Bucher et al., 1980, *J Virol* 36:586-590).
- 11- (Francis et al., 1987, *J Gen Virol* 68:2687-2691).
- 12- (Morein et al., 1983, *J Gen Virol* 64:1557-1569).
- 13- (Garbe et al., 1994, *Infect Immun* 62:3092-3101).

In another preferred embodiment, a microbial pathogen may include a pathogen causing a sexually transmitted disease selected from the group of pathogens, with respective antigens, in Table 3.

Table 3

PATHOGEN	INFECTION/DISEASE	PROTEIN ANTIGEN
35 <i>N. gonorrhoeae</i>	gonorrhea	IgA1 protease ¹ , PIB ² , H.8 ³ , Por ⁴
<i>Chlamydia trachomatis</i>	nongonococcal urethritis	MOMP ⁵ , HSP ⁶

- 1- (Lomholt et al., 1994, *Infect Immun* 62:3178-83).
- 2- (Heckels et al., 1990, *Vaccine* 8:225-230).
- 3- (Blacker et al., 1985, *J Infect Dis* 151:650-657).
- 4- (Wetzler et al., 1992, *Vaccine* 8:225-230).
- 5- (Campos et al., 1995, *Ophthalmol Vis Sci* 36:1477-91; Murdin et al., 1995, *Infect Immun* 63:1116-21).
- 45 6- (Taylor et al., 1990, *Infect Immun* 58:3061-3).

Tables 2 & 3, and the references footnoted which are herein incorporated by reference, illustrate various protein antigens, or peptides thereof, viewed by those skilled in the art to be useful as vaccine candidates against the respective microbial pathogen. Typically, the immunopotency of an epitope, whether from a protein or peptide, of a microbial pathogen is determined by monitoring the immune response of an animal following immunization with the epitope and/or by analyzing human convalescent sera in conjunction with pre-immune sera. Thus, one skilled in the art can determine protein or peptide antigens from microbial pathogens which would be desired to include as a heterologous antigen to be expressed by an *htrB* mutant according to the present invention. A corresponding nucleic acid sequence, the encoding sequence, can then be deduced from the amino acid sequence of the protein or peptide antigen, wherein the encoding sequence is introduced into the *htrB* mutant for expression.

EXAMPLE 14

Use of *htrB* mutants to generate antisera

The *htrB* mutant, or endotoxin purified therefrom, can be used to generate endotoxin-specific antisera, directed to the particular gram-negative bacterial pathogen, which can be used in an immunoassay to detect the antigen (that particular gram-negative bacterial pathogen), present in the body fluid of an individual suspected of having an infection caused by that gram-negative bacterial pathogen. The body fluid(s) collected for analysis depend on the microorganism to be detected, the suspected site of infection, and whether the body fluid is suspected of containing the antigen or containing antisera. With those considerations in mind, the body fluid could include one or more of sputum, blood, cerebrospinal

fluid, lesion exudate, swabbed material from the suspected infection site, and fluids from the upper respiratory tract. Immunoassays for such detection comprises any immunoassay known in the art including, 5 but not limited to, radioimmunoassay, ELISA, "sandwich" assay, precipitin reaction, agglutination assay, fluorescent immunoassay, and chemiluminescence-based immunoassay.

Alternatively, where an immunocompromised 10 individual is suffering from a potentially life-threatening infection caused by a particular gram-negative bacterial pathogen, immunization may be passive, i.e. immunization comprising administration of purified human immunoglobulin containing antibody 15 against an *htrB* mutant or isolated *htrB* endotoxin of that particular gram-negative bacterial pathogen.

It should be understood that while the invention has been described in detail herein, the 20 examples were for illustrative purposes only. Other modifications of the embodiments of the present invention that are obvious to those skilled in the art of molecular biology, medical diagnostics, and related disciplines are intended to be within the scope of the 25 appended claims.

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- 5 (i) APPLICANT: Michael A. Apicella
Melvin G. Sunshine
Na-Gyong Lee
Bradley Gibson
Rasappa Arumugham
- 10 (ii) TITLE OF INVENTION: Non-Toxic Mutants of Pathogenic
Gram-Negative Bacteria
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
- 15 (A) ADDRESSEE: Hodgson, Russ, Andrews, Woods &
Goodyear
- (B) STREET: 1800 One M&T Plaza
- (C) CITY: Buffalo
- (D) STATE: New York
- 20 (E) COUNTRY: United States
- (F) ZIP: 14203-2391
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Diskette, 3.5 inch, 1.4 Mb
storage
- 25 (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: MS-DOS/ Microsoft Windows
3.1
- (D) SOFTWARE: Wordperfect for Windows 5.1
- (vi) APPLICATION DATA:
- 30 (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (vii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Nelson, M. Bud
- (B) REGISTRATION NUMBER: 35,300
- 35 (C) REFERENCE DOCKET NUMBER: 22244.0002
- (viii) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: (716) 856-4000
- (B) TELEFAX: (716) 849-0349
- 40 (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 969 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double-stranded
- 45 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: genomic DNA
- (iii) HYPOTHETICAL: yes
- (iv) ORIGINAL SOURCE:
- (A) ORGANISM: *H. influenzae*
- 50 (B) STRAIN: 2019
- (C) CELL TYPE: bacterium
- (v) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TAAACTACGC CCCTAACTTA CGTGGAAAGA ACA ATG AAA AAC 42

- 61 -

Met Lys Asn
1

5	GAA Glu	AAA Lys	CTC Leu	CCT Pro	CAA Gln	TTT Phe	CAA Gln	CCG Pro	CAC His	TTT Phe	TTA Leu	GCC Ala	CCA Pro	81
		5					10					15		
10	AAA Lys	TAC Tyr	TGG Trp	CTT Leu	TTT Phe	TGG Trp	CTA Leu	GGC Gly	GTG Val	GCA Ala	ATT Ile	TGG Trp	CGA Arg	120
				20					25					
15	AGT Ser	ATT Ile	TTA Leu	TGT Cys	CTT Leu	CCC Pro	TAT Tyr	CCT Pro	ATT Ile	TTG Leu	CGC Arg	CAT His	ATT Ile	159
	30					35					40			
20	GGT Gly	CAT His	GGT Gly	TTC Phe	GGT Gly	TGG Trp	CTG Leu	TTT Phe	TCA Ser	CAT His	TTA Leu	AAA Lys	GTG Val	198
			45					50					55	
25	GGT Gly	AAA Lys	CGT Arg	CGA Arg	GCT Ala	GCC Ala	ATT Ile	GCA Ala	CGC Arg	CGT Arg	AAT Asn	CTT Leu	GAA Glu	237
					60					65				
30	CTT Leu	TGT Cys	TTC Phe	CCT Pro	GAT Asp	ATG Met	CCT Pro	GAA Glu	AAC Asn	GAA Glu	CGT Arg	GAG Glu	ACG Thr	276
		70					75					80		
35	ATT Ile	TTG Leu	CAA Gln	GAA Glu	AAT Asn	CTT Leu	CGT Arg	TCA Ser	GTA Val	GGC Gly	ATG Met	GCA Ala	ATT Ile	315
			85						90				95	
40	ATC Ile	GAA Glu	ACT Thr	GGC Gly	ATG Met	GCT Ala	TGG Trp	TTT Phe	TGG Trp	TCG Ser	GAT Asp	TCA Ser	CGT Arg	354
						100					105			
45	ATC Ile	AAA Lys	AAA Lys	TGG Trp	TCG Ser	AAA Lys	GTT Val	GAA Glu	GGC Gly	TTA Leu	CAT His	TAT Tyr	CTA Leu	393
			110					115					120	
50	AAA Lys	GAA Glu	AAT Asn	CAA Gln	AAA Lys	GAT Asp	GGA Gly	ATT Ile	GTT Val	CTC Leu	GTC Val	GGC Gly	GTT Val	432
					125					130				
55	CAT His	TTC Phe	TTA Leu	ACG Thr	CTA Leu	GAA Glu	CTT Leu	GGC Gly	GCA Ala	CGC Arg	ATC Ile	ATT Ile	GGT Gly	471
		135					140					145		
60	TTA Leu	CAT His	CAT His	CCT Pro	GGC Gly	ATT Ile	GGT Gly	GTT Val	TAT Tyr	CGT Arg	CCA Pro	AAT Asn	GAT Asp	510
				150					155					
65	AAT Asn	CCT Pro	TTG Leu	CTT Pro	GAT Asp	TGG Trp	CTA Leu	CAA Gln	ACA Thr	CAA Gln	GGC Gly	CGT Arg	TTA Leu	549
	160					165					170			

	CGC	TCC	AAT	AAA	GAT	ATG	CTT	GAT	CGT	AAA	GAT	TTA	CGC	588
	Arg	Ser	Asn	Lys	Asp	Met	Leu	Asp	Arg	Lys	Asp	Leu	Arg	
			175					180					185	
5	GGA	ATG	ATC	AAA	GCT	TTA	CGC	CAC	GAA	GAA	ACC	ATT	TGG	627
	Gly	Met	Ile	Lys	Ala	Leu	Arg	His	Glu	Glu	Thr	Ile	Trp	
				190					195					
10	TAT	GCG	CCT	GAT	CAC	GAT	TAC	GGC	AGA	AAA	AAT	GCC	GTT	666
	Tyr	Ala	Pro	Asp	His	Asp	Tyr	Gly	Arg	Lys	Asn	Ala	Val	
		200					205					210		
15	TTT	GTT	CCT	TTT	TTT	GCA	GTA	CCT	GAC	ACT	TGC	ACT	ACT	705
	Phe	Val	Pro	Phe	Phe	Ala	Val	Pro	Asp	Thr	Cys	Thr	Thr	
				215					220					
20	ACT	GGT	AGT	TAT	TAT	TTA	TTG	AAA	TCC	TCG	CAA	AAC	AGC	744
	Thr	Gly	Ser	Tyr	Tyr	Leu	Leu	Lys	Ser	Ser	Gln	Asn	Ser	
	225					230					235			
25	AAA	GTG	ATT	CCA	TTT	GCG	CCA	TTA	CGC	AAT	AAA	GAT	GGT	783
	Lys	Val	Ile	Pro	Phe	Ala	Pro	Leu	Arg	Asn	Lys	Asp	Gly	
			240					245					250	
30	TCA	GGC	TAT	ACC	GTG	AGC	ATT	TCA	GCG	CCT	GTT	GAT	TTT	822
	Ser	Gly	Tyr	Thr	Val	Ser	Ile	Ser	Ala	Pro	Val	Asp	Phe	
					255					260				
35	ACA	GAT	TTA	CAA	GAT	GAA	GTA	GCG	ATA	GCT	GTG	CGA	ATG	861
	Thr	Asp	Leu	Gln	Asp	Glu	Val	Ala	Ile	Ala	Val	Arg	Met	
		265				270						275		
40	AAT	CAA	ATC	GTT	GAA	AAG	GAA	ATC	ATG	AAG	GGC	ATA	TCA	900
	Asn	Gln	Ile	Val	Glu	Lys	Glu	Ile	Met	Lys	Gly	Ile	Ser	
				280					285					
45	CAA	TAT	ATG	TGG	CTA	CAT	CGT	CGT	TTT	AAA	ACA	CGC	CCC	
	Gln	Tyr	Met	Trp	Leu	His	Arg	Arg	Phe	Lys	Thr	Arg	Pro	
	290					295					300			
50	GAT	GAA	AAT	ACG	CCT	AGT	TTA	TAC	GAT	TAA				969
	Asp	Glu	Asn	Thr	Pro	Ser	Leu	Tyr	Asp					
			305					310						

- (3) INFORMATION FOR SEQ ID NO:2:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 31 nucleotides
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single-stranded
 - (D) TOPOLOGY: linear
- (ii) ORIGINAL SOURCE:
- (A) ORGANISM: *H. influenzae*
 - (B) STRAIN: 2019

(iii) IMMEDIATE SOURCE: synthesized
(iv) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCAATATGGC GCAAAATAGG ATAGGGAAGA C 31

5

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 nucleotides
(B) TYPE: nucleic acid
10 (C) STRANDEDNESS: single-stranded
(D) TOPOLOGY: linear

(ii) ORIGINAL SOURCE:

(A) ORGANISM: *H. influenzae*

(iii) FEATURE:

- 15 (A) OTHER INFORMATION: uptake sequence for transformation

(iv) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAGTGCGGT 9

20

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
25 (C) STRANDEDNESS: single-stranded
(D) TOPOLOGY: linear

(ii) FEATURE:

(A) OTHER INFORMATION: hybridizes to TNF α mRNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:4:

30

ATCTCTCAGC TCCACGCCAT TGGCCAGGAG 30

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single-stranded
(D) TOPOLOGY: linear

(ii) FEATURE:

- 40 (A) OTHER INFORMATION: does not hybridize to TNF α mRNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCCTGGCCA ATGGCGTGGA GCTGAGAGAT 30

What is claimed is:

- 5 1. A method for immunizing an individual to prevent
disease caused by a gram-negative bacterial pathogen,
the method comprising vaccinating the individual with a
prophylactically effective amount of a vaccine
formulation comprising an active ingredient selected
10 from the group consisting of an *htrB* mutant of said
gram-negative bacterial pathogen, endotoxin isolated
from the *htrB* mutant of said gram-negative bacterial
pathogen, endotoxin isolated from the *htrB* mutant of
said gram-negative bacterial pathogen said endotoxin
15 conjugated to a carrier protein, and an *htrB* mutant of
said gram-negative bacterial pathogen which has been
genetically engineered to express at least one
heterologous vaccine antigen; wherein said *htrB* mutant
lacks one or more secondary acyl chains of lipid A
20 contained in the gram-negative bacterial pathogen
resulting in substantially reduced toxicity when
compared to lipid A of the gram-negative bacterial
pathogen.
- 25 2. The method of claim 1, wherein the individual is a
human, and the vaccine formulation is introduced by a
route of administration selected from the group
consisting of intradermal, intramuscular,
intraperitoneal, intravenous, subcutaneous, ocular,
30 intranasal, and oral administration.
3. The method of claim 2, wherein the vaccine
formulation comprises an active ingredient consisting
essentially of an *htrB* mutant of said gram-negative
35 bacterial pathogen.

4. The method of claim 2, wherein the vaccine formulation comprises an active ingredient consisting essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen.

5

5. The method of claim 2, wherein the vaccine formulation comprises an active ingredient consisting essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, wherein the isolated endotoxin is conjugated to a carrier protein.

10

6. The method of claim 2, wherein the vaccine formulation comprises an active ingredient consisting essentially of an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous antigen from a microbial pathogen.

15

7. The method of claim 2, wherein the vaccine formulation further comprises a physiological carrier and an adjuvant.

20

8. The method of claim 1, wherein the individual is an animal, and the vaccine formulation is introduced by a route of administration selected from the group consisting of intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, ocular, intranasal, and oral administration.

25

9. The method of claim 8, wherein the vaccine formulation comprises an active ingredient consisting essentially of an *htrB* mutant of said gram-negative bacterial pathogen.

30

10. The method of claim 8, wherein the vaccine formulation comprises an active ingredient consisting

35

essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen.

11. The method of claim 8, wherein the vaccine
5 formulation comprises an active ingredient consisting essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, wherein the isolated endotoxin is conjugated to a carrier protein.
12. The method of claim 8, wherein the vaccine
10 formulation comprises an active ingredient consisting essentially of an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous antigen from a
15 microbial pathogen.
13. The method according to claim 8, wherein the *htrB*
mutant of said gram-negative bacterial pathogen is administered orally as an additive to animal feed.
20
14. The method according to claim 12, wherein the *htrB*
mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous antigen from a microbial pathogen is
25 administered orally as an additive to animal feed.
15. The method of claim 8, wherein the vaccine
formulation further comprises a physiological carrier and an adjuvant.
30
16. A vaccine formulation comprising an active
ingredient selected from the group consisting of an *htrB*
mutant of a gram-negative bacterial pathogen, endotoxin
isolated from the *htrB* mutant of said gram-negative
35 bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen said

endotoxin conjugated to a carrier protein, and an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at least one heterologous vaccine antigen; wherein said *htrB* mutant
5 lacks one or more secondary acyl chains of lipid A contained in the gram-negative bacterial pathogen resulting in substantially reduced toxicity when compared to lipid A of the gram-negative bacterial pathogen.

10

17. The vaccine formulation according to claim 16, wherein the active ingredient consists essentially of an *htrB* mutant of said gram-negative bacterial pathogen.

15

18. The vaccine formulation according to claim 16, wherein the active ingredient consists essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen.

20

19. The vaccine formulation of claim 16, wherein the active ingredient consists essentially of endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen, wherein the isolated endotoxin is conjugated to a carrier protein.

25

20. The vaccine formulation according to claim 16, wherein the active ingredient consists essentially of an *htrB* mutant of said gram-negative bacterial pathogen which has been genetically engineered to express at
30 least one heterologous antigen from a microbial pathogen.

21. The vaccine formulation according to claim 16, further comprising a physiological carrier and an
35 adjuvant.

22. A method of making in a gram-negative bacterial pathogen a mutant endotoxin of substantially reduced toxicity when compared to the endotoxin of the wild type gram-negative bacterial pathogen, the method comprising
5 mutating an *htrB* gene within the gram-negative bacterial pathogen, wherein said mutation causes a phenotype of a resultant *htrB* mutant characterized by a mutant endotoxin lacking one or more secondary acyl chains of lipid A contained in the wild type gram-negative
10 bacterial pathogen.

23. A mutant endotoxin of substantially reduced toxicity, made according to the method of claim 22, wherein the mutant endotoxin having substantially
15 reduced toxicity was purified from the *htrB* mutant by a process selected from the group consisting of a phenol/water extraction, and a protease digestion; and wherein the purified mutant endotoxin having substantially reduced toxicity is used to generate
20 endotoxin-specific antibodies.

24. The mutant endotoxin according to claim 23, further comprising conjugation to a carrier protein.

25. A mutant endotoxin of substantially reduced toxicity, made according to the method of claim 22.

26. The mutant endotoxin according to claim 25, further comprising conjugation to a carrier protein.
30

27. A method of making an *htrB* mutant of a wild type gram-negative bacterial pathogen wherein the *htrB* mutant has substantially reduced toxicity when compared to the wild type gram-negative bacterial pathogen, the method
35 comprising mutating an *htrB* gene within the gram-negative bacterial pathogen, wherein said mutation

causes a phenotype of a resultant *htrB* mutant characterized by endotoxin lacking at least one secondary acyl chain on lipid A contained in the wild type gram-negative bacterial pathogen.

5

28. A mutant gram-negative bacterial pathogen of substantially reduced toxicity, made according to the method of claim 27, wherein the gram-negative bacterial pathogen having substantially reduced toxicity is used
10 to generate endotoxin-specific antibodies.

29. A method for producing endotoxin-specific antisera for a use selected from the group consisting of in diagnostic assays, and for passive immunization, the
15 method comprises immunizing an individual with a vaccine formulation comprising an active ingredient selected from the group consisting of an *htrB* mutant of said gram-negative bacterial pathogen, endotoxin isolated from the *htrB* mutant of said gram-negative bacterial
20 pathogen, and endotoxin isolated from the *htrB* mutant of said gram-negative bacterial pathogen said endotoxin conjugated to a carrier protein; and collecting antibody produced from said immunized individual; wherein said *htrB* mutant lacks one or more secondary acyl chains of
25 lipid A contained in the wild type gram-negative bacterial pathogen resulting in substantially reduced toxicity when compared to lipid A of the wild type gram-negative bacterial pathogen.

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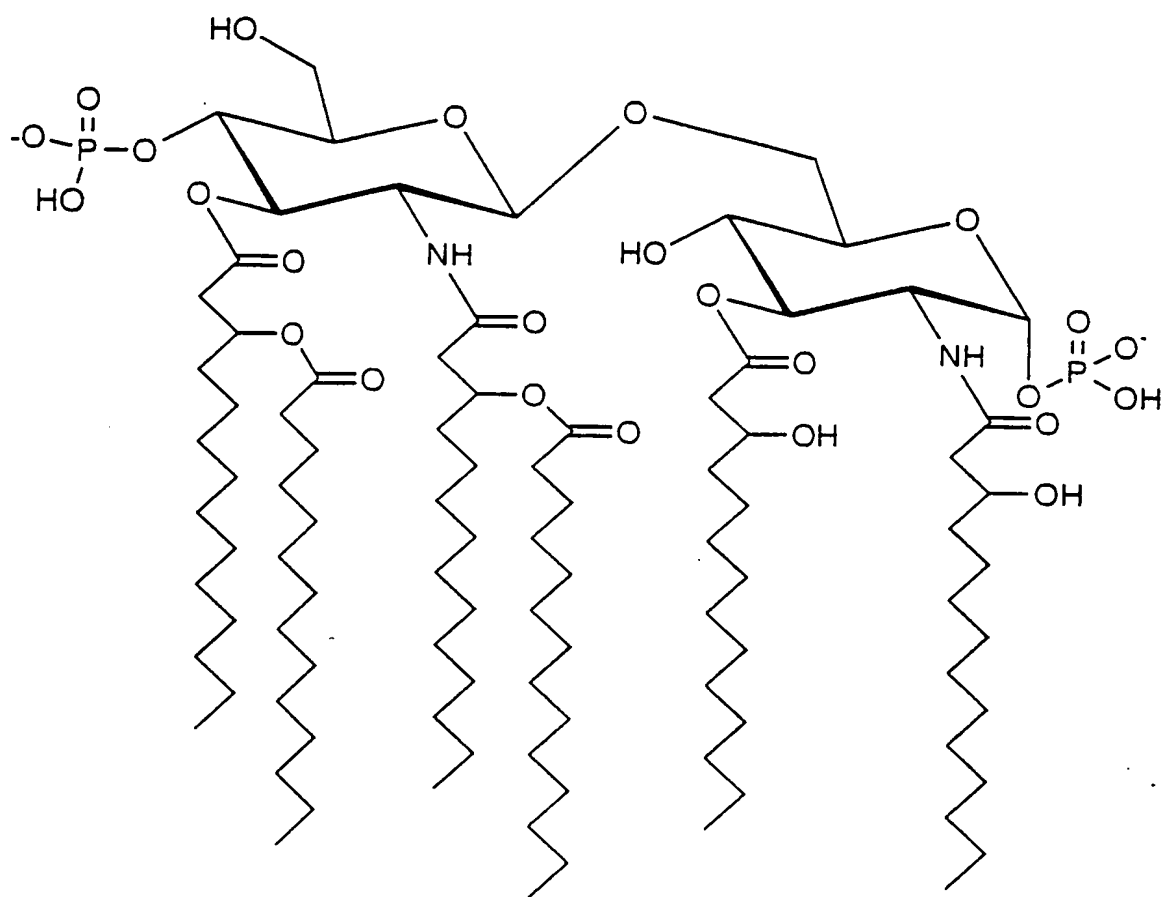
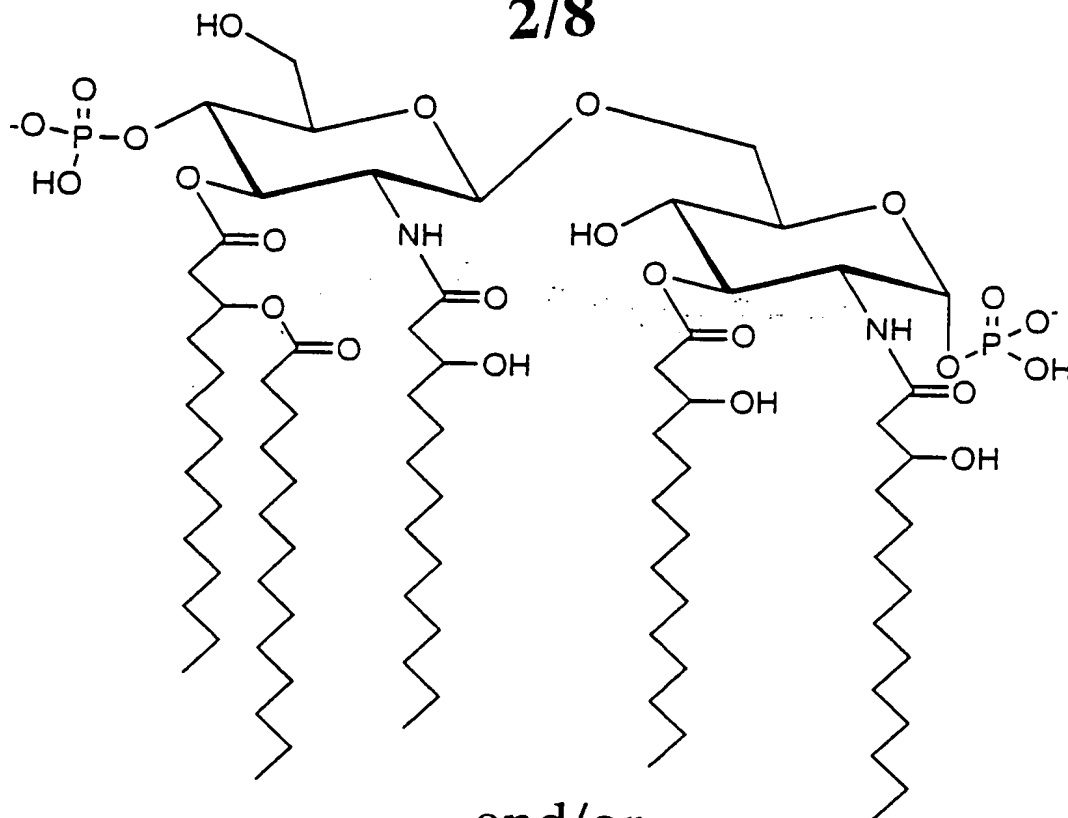


FIG. 1

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and/or

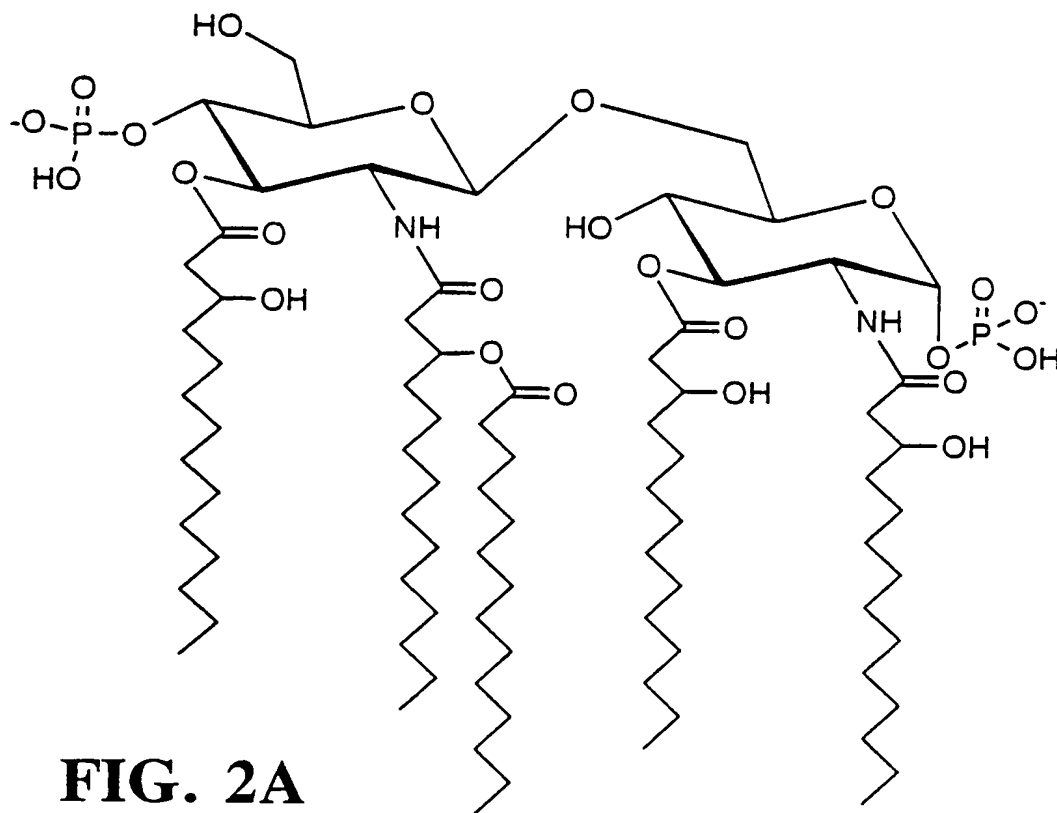


FIG. 2A

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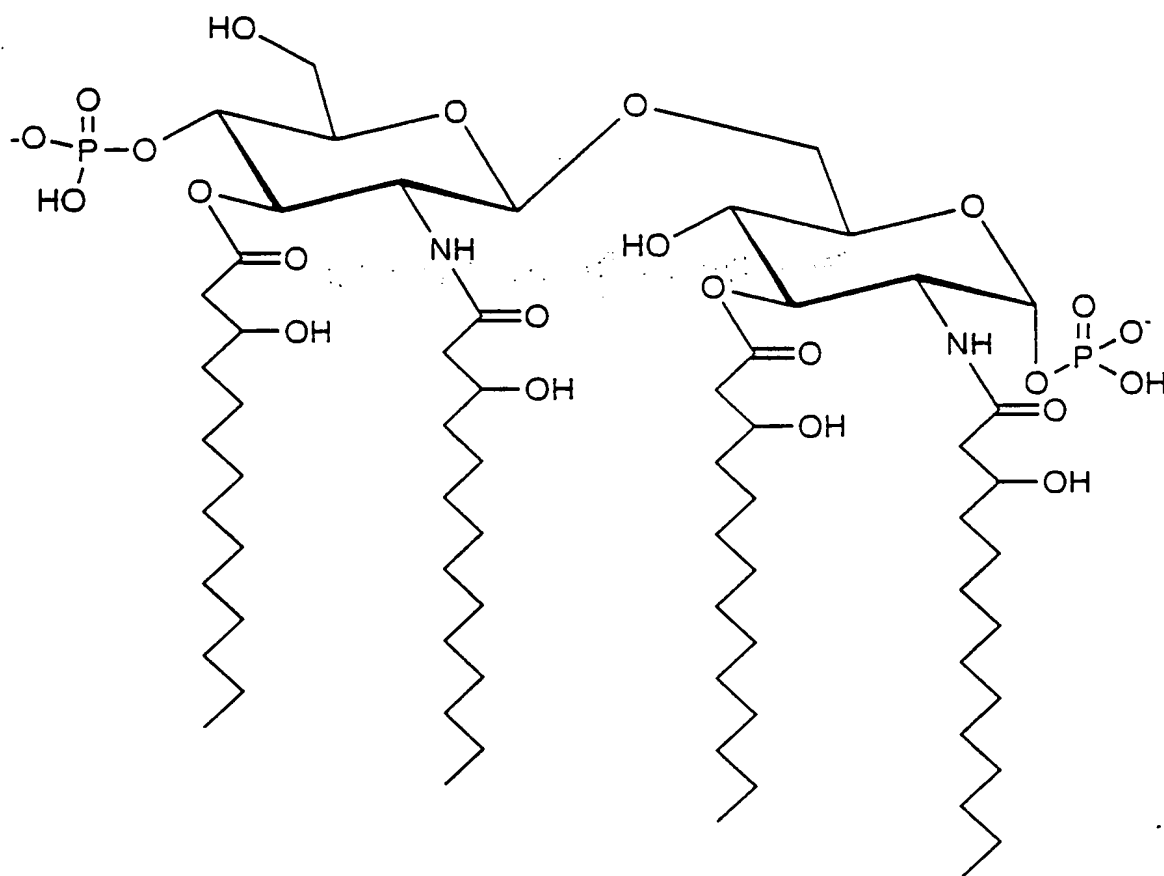


FIG. 2B

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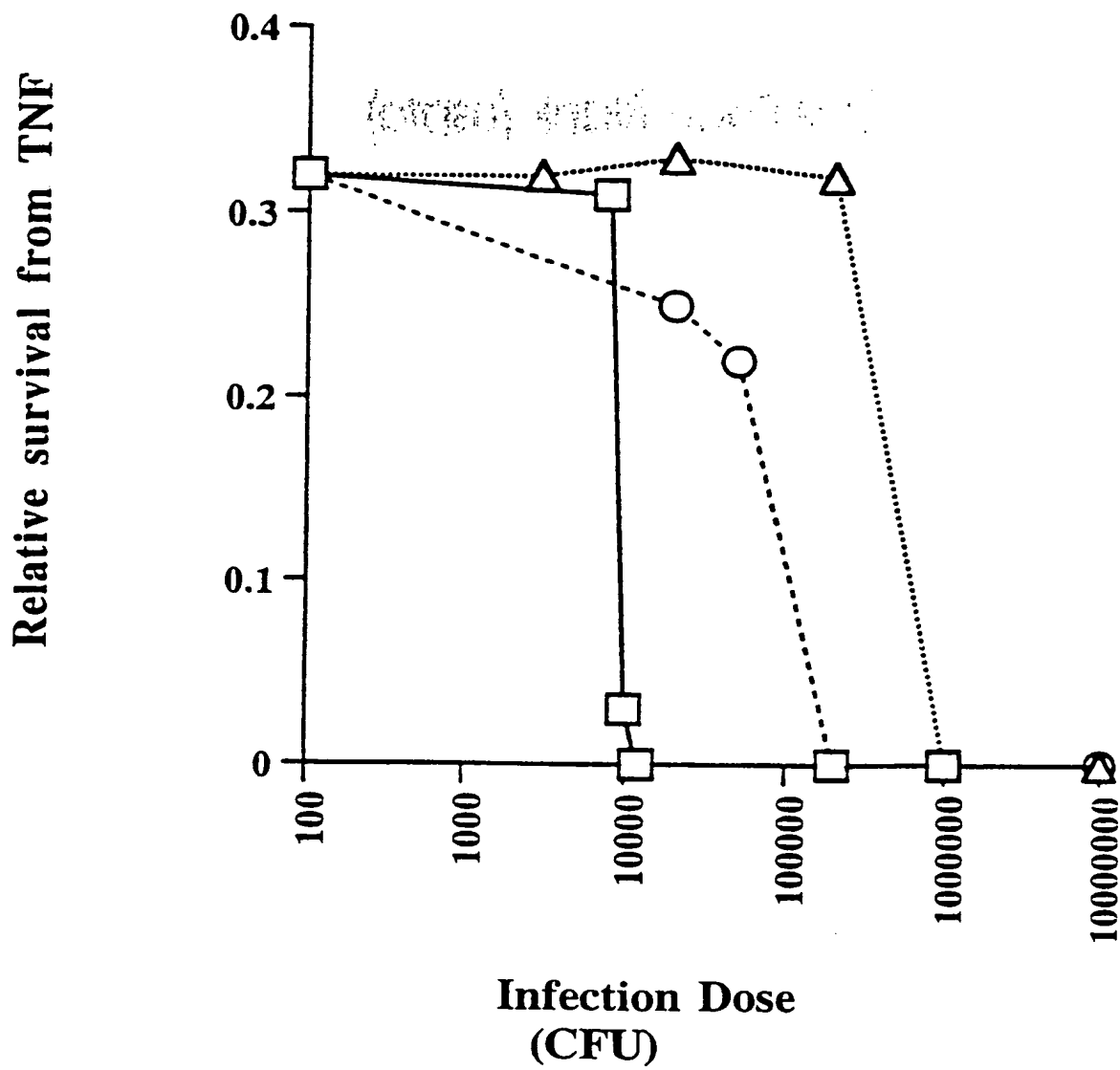


FIG. 3

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Unstimulated cells
Probe #1



Unstimulated cells
No probe



1 ug/ml 29-LOS
Probe #2

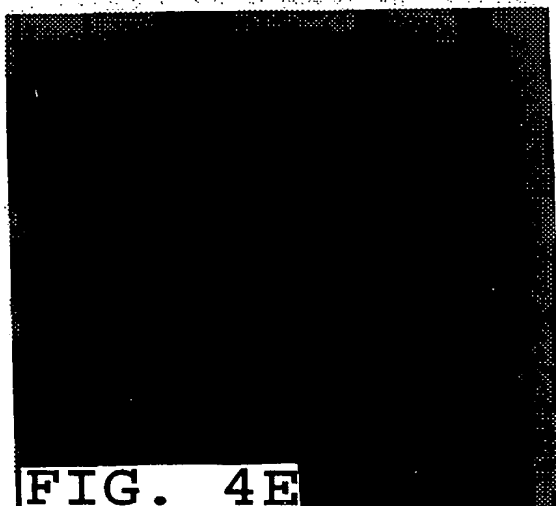


FIG. 4E

1 ug/ml 2019-LOS
Probe #1

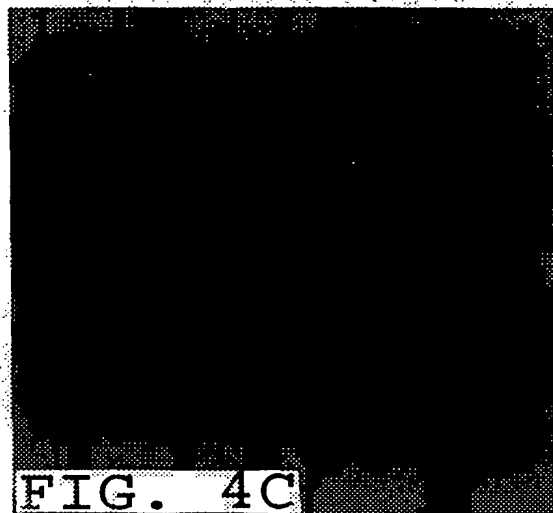


FIG. 4C

1 ug/ml 2019-LOS
Probe #2



FIG. 4D

1 ug/ml B29-LOS
Probe #1

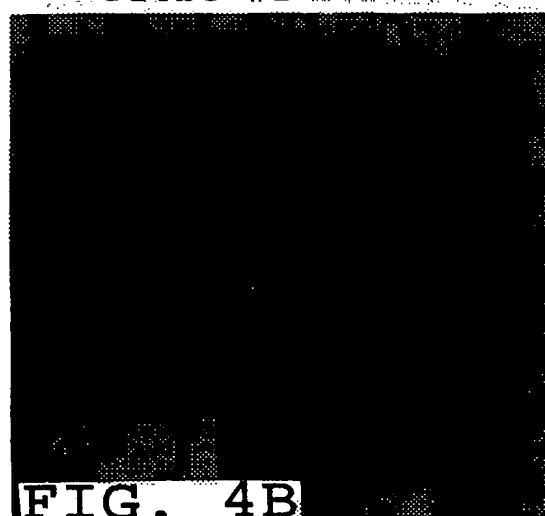


FIG. 4B

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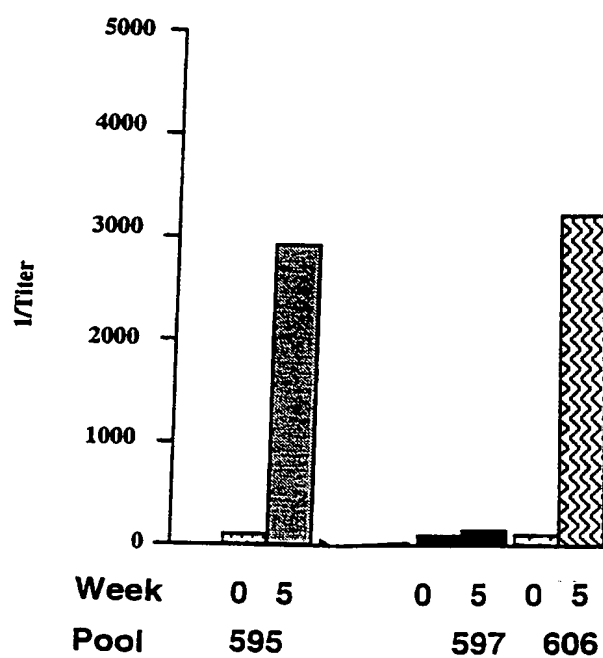


FIG. 5

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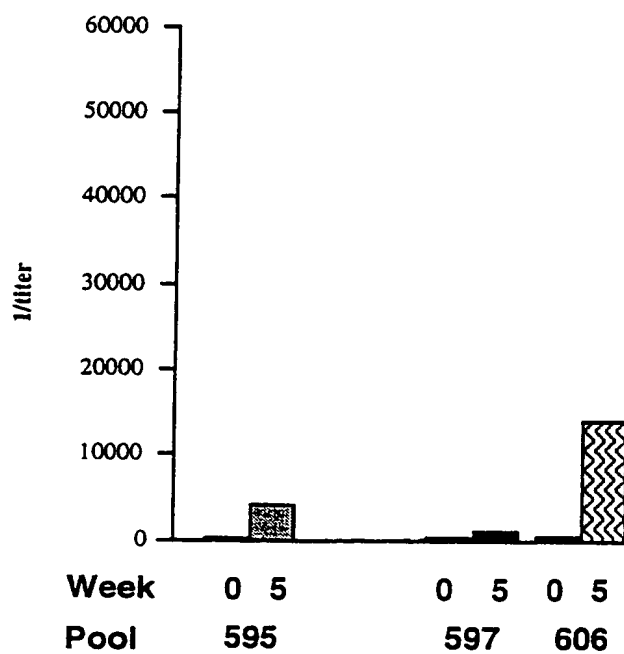


FIG. 6

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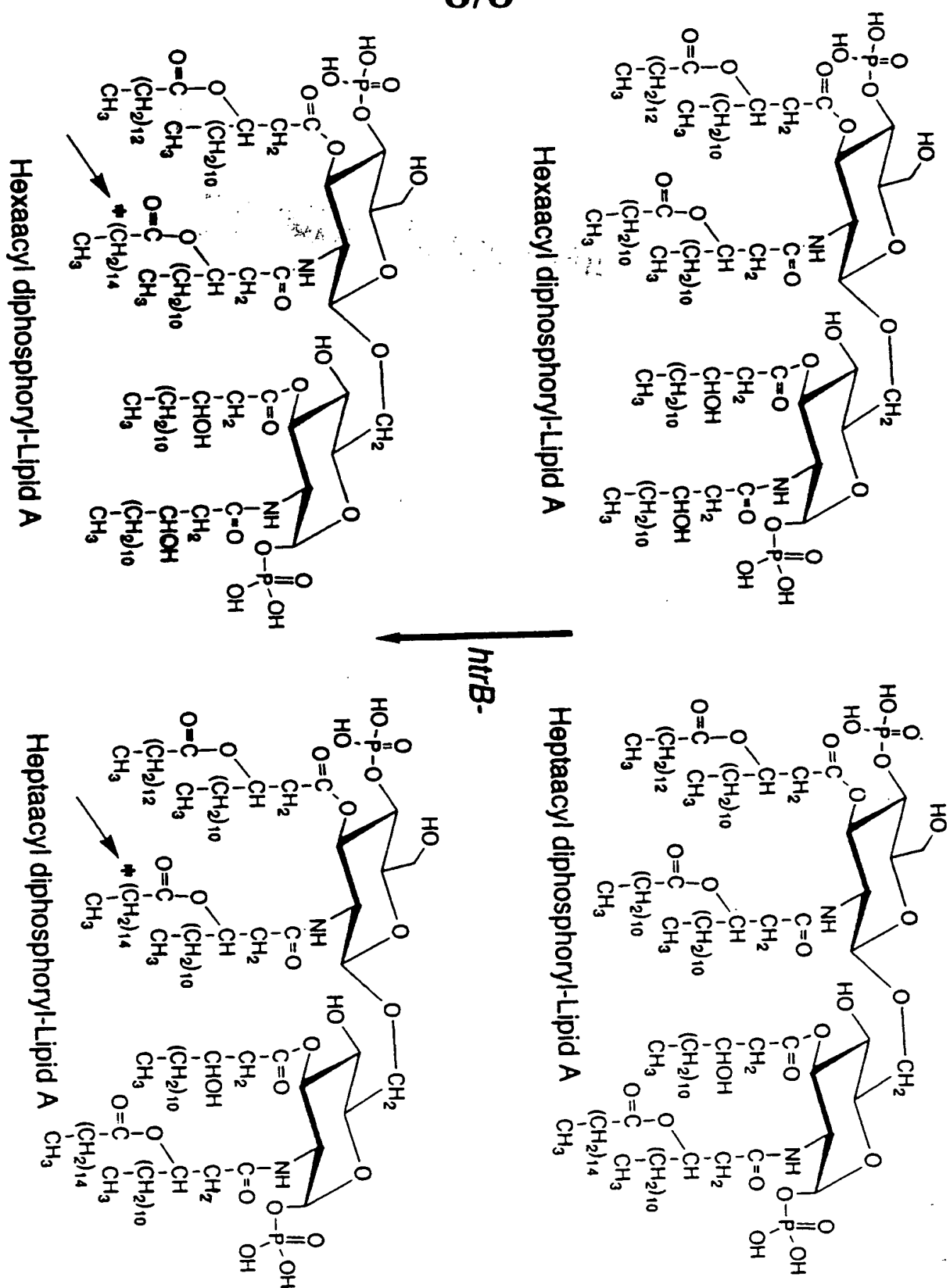


FIG. 7

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INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/US96/18984
A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 35/66, 39/00, 39/02; C07K 14/195

US CL : 424/9.2, 184.1, 234.1, 236.1; 530/403, 825

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.2, 184.1, 234.1, 236.1; 530/403, 825

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, MEDLINE, BIOSIS, HCAPLUS, WPIDS, EMBASE

search terms: endotoxin, gram-negative bacteria, htrb mutant, vaccine, pathogen

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,707,543 (ZOLLINGER et al.) 17 November 1987, see entire document.	1-16
Y	US, A, 4,912,094 (MYERS et al.) 27 March 1990, see entire document.	16-21
X	US, A, 4,929,604 (MUNFORD et al.) 29 May 1990, see entire document.	16-21
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Y		22-29
A	ZIEGLER et al. Treatment of Gram-Negative Bacteremia and Shock With Antiserum to a Mutant Escherichia Coli. 11 November 1982. Volume 307, Number 20, pages 1225-1230, see entire document.	1-29



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

&

document member of the same patent family

Date of the actual completion of the international search

30 JANUARY 1997

Date of mailing of the international search report

25 MAR 1997

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